Wang, Xiao-Fan

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GRANT NUMBER DAMD17-94-J-4065

TITLE: The Roles of TGF-Beta and TGF-Beta Signaling Receptors in Breast Carcinogenesis

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REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1997	Annual (1 Jul 96 - 30 Jun 97)

4. TITLE AND SUBTITLE
The Roles of TGF-Beta and TGF-Beta Signaling Receptors in Breast Carcinogenesis

5. FUNDING NUMBERS
DAMD17-94-J-4065

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The overall goal of this research project is to explore the roles of TGF-ß and components of its signaling pathways in the initiation, progression and metastasis of breast adenocarcinomas through an investigation of the disregulation of TGF-ß signal transduction. Last year, we identified and isolated three cDNAs encoding members of the Smad family and studied the TGF-ß induced phosphorylation of these molecules in a normal mammary epithelial cell line. Subsequently, we have focused on the functional role of Smad3 and Smad4 as tumor suppressors in mediating the TGF-ß signal in transactivating downstream target genes. We have also continued our investigation on the mechanism by which TGF-ß activates the expression of cyclin-dependent kinase inhibitor p15 gene in MCF7 cells as well as the importance of a paracrine loop mediated by the interactions between mammary epithelial and fibroblst cells. Results from further analysis in these directions will not only significantly contribute to an understanding of the molecular events leading to breast carcinogenesis, but also aid in the development of new therapeutics for breast cancer.

14. SUBJECT TERMS
Breast Cancer, TGF-beta, Signal Transduction

15. NUMBER OF PAGES
70

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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7/7/97

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A. INTRODUCTION

Breast cancer is the most common cancer in women in the United States. Endocrine therapy has proven to be beneficial in approximately one-third of breast cancer patients. However, the tumors inevitably progress to a state of hormone insensitivity and no longer respond to conventional endocrine therapies. Therefore, it is necessary to identify other molecular markers to monitor the pathological process of the disease in order to better evaluate patient prognosis and to elucidate molecular mechanism of breast cancer initiation, progression and metastasis in order to develop new reagents for subsequent treatments. The proposed study of TGF-ß growth inhibitory signaling pathways in breast carcinogenesis will significantly benefit both of these purposes.

The overall goal of this research project has been to explore the roles of TGF-ß and components of its signaling pathways in the initiation, progression and metastasis of breast adenocarcinomas through an investigation of the disregulation of TGF-ß signal transduction. While breast cancers almost exclusively originate in epithelial cells, they cannot develop significantly without manipulating and recruiting the activities of surrounding vasculature, matrix proteins, and stromal components. Of particular importance to the genesis and progression of breast cancers are the stromal/epithelial interactions, as these interactions have been found to be important to both neoplastic and normal development of mammary tissue (10). Normal growth of breast epithelium is tightly regulated by a complex system of negative and positive autocrine and paracrine controls. Involved in this control are the growth promoting factors such as the hormones, estrogen and progesterone, the IGFs, and the growth inhibiting factors such as the TGF-ßs. Although it is known that tissue growth homeostasis is maintained by communication between the stromal and epithelial cells, the molecular details of these interactions are poorly understood. It is clear, however, that aberration of this homeostasis can lead to the development of a neoplastic state.

TGF-ßs are a group of multifunctional polypeptide hormones which play important roles in many normal cellular functions including the regulation of proliferation, differentiation, extracellular matrix formation, cell adhesion and migration (32,40). Additionally, TGF-ß acts to inhibit the proliferation of a variety of cell types, including breast epithelium, by playing a pivotal role in maintaining negative autocrine and paracrine loops (45). Therefore, lesions to TGF-ß signaling pathways that disrupt the negative growth regulation of breast epithelia may contribute to mammary carcinogenesis and represent an obligatory step in neoplastic progression of breast epithelia. Indeed, loss of TGF-ß responsiveness of MCF-7 breast cancer cells has been shown to correlate with a loss of expression in these cells of the TGF-ß signaling receptors (46). Loss of such autocrine control by TGF-ß represents an opportunity for malignant epithelia to increase proliferation in response to other positive growth factors, like IGF, and hormones, like estrogen.

Elucidation of signaling mechanism by the TGF-ß receptor complex and the discovery of a molecular link between the TGF-ß signal and the cell cycle control machinery have significantly advanced our knowledge of the molecular nature of the TGF-ß growth-inhibitory signaling pathway. However, the intracellular signaling cascade through which TGF-ß signals remains largely unknown. A number of molecules which interact with the receptor kinases and serve as in vitro substrates have been isolated through the two-hybrid system in yeast (6,21,51,52). The functional significance of those interactions, however, remains mostly unknown since the phosphorylation events have not been observed to be regulated by TGF-ß in vivo and there is no indication of how these interacting molecules could potentially be involved in TGF-ß signaling.

Genetic analyses in Drosophila and in the nematode C. elegans have led to the isolation of TGF-ß-like pathway components, including ligands and receptors which are homologous to those identified in vertebrates (24). In Drosophila, DPP, the best characterized gene implicated in the regulation of cell fate throughout fly development, is a member of the TGF-ß superfamily (37,50). The function of DPP in Drosophila can be substituted by its most closely related mammalian

homologs in the family, BMP2 and BMP4 (33). Recently, a gene termed MAD (Mothers against dpp) was isolated in a screen for modifiers of DPP activity (44). Functional studies demonstrated that MAD is required for DPP activity in many aspects of development and it acts downstream of the DPP receptor (35,53). Similar studies in C. elegans resulted in the isolation of three MAD homologs, SMA-2, SMA-3, and SMA-4, which potentially serve as downstream effectors in the TGF-ß-like signaling pathway in that organism, since mutations in these genes cause the same mutant phenotype as the mutant receptors of the TGF-ß-like ligands (41,43). Evidence which further supports the notion that the MAD and SMAs (this group of molecules have recently been renamed Smads (12)) are involved in the signaling pathways for the family of TGF-ß ligands came from the isolation of Smad homologs in Xenopus, mouse, and human (33).

To this end, we and others have demonstrated that Smads undergo phosphorylation and accumulate in the nucleus in response to TGF-ß and its family members (2,14,19,27,29,30). The phosphorylation is catalyzed by the type I receptor kinases and the phosphorylation sites have been mapped to a few serine residues in the C-terminal portion of Smad1 and Smad2, though the corresponding highly conserved sites in Smad3 and Smad5 most likely serve the same function as recipient residues for phosphorylation (25,30,56). Based on available evidence on the kinase-substrate specificity demonstrated so far, it appears that Smad2 and Smad3, which share 92% sequence identity, are likely mediators for the TGF-ß signal, whereas Smad1 and Smad5, which share the same high level of sequence identity, act as effectors for the BMPs, members of the TGF-ß superfamily. Smad4, on the other hand, is shared as a partner for the formation of heteromeric complexes with the other members of the Smad family (26). This line-up of specificity is consistant with that defined in the Xenopus system (2,15,26,47). At the functional level, genetic analyses performed in humans suggest that the Smad proteins may play important roles in the inhibition of cell proliferation, a hallmark of TGF-ß function. Genes encoding two members of the Smad family, DPC4 (Smad4) and MADR2 (Smad2) have been localized to chromosome 18q21 in humans and found to be either deleted or mutated in a majority of human pancreatic cancers, certain colon carcinomas, and a variety of other types of human cancers including breast cancer (3,14,17,23,34,39,42,48), suggesting that the Smads function as tumor suppressor genes. At the molecular level, the Smads may function to participate in transcriptional activation once they enter the nucleus, since it has been observed that the C-terminal domains of a number of Smads display transcriptional activity when bound to DNA via a GAL4 DNA-binding domain (29). This activity is eliminated by hot spot nonsense or missense mutations found in various Smad alleles, including a few mutant Smad4/DPC4 and Smad2/MADR2 alleles associated with human pancreatic and colon cancers (14,29), evidence strongly suggesting that the transcriptional activity of Smads is directly linked to their tumor suppressing or growth inhibitory activities. It is important to point out that the tumor suppressing activity of Smads could well be also associated with their involvement of regualtion of cell-cell and cell-matrix interactions as disruption in these biological processes could contribute to tumor growth.

In a separate study, the wild type Smad3 and Smad4 were found to activate transcription synergistically, in a TGF-ß independent fashion, from the promoter of plasminogen activator inhibitor-1 (PAI-1) gene, a known target for the TGF-ß mediated regulation of extracellular matrix formation (56). As discussed in more detail below, we have extended this type of study further to show that Smad3/Smad4 complex could physically interact with specific DNA response elements in both the PAI-1 and collagenase promoters. Though we have demonstrated that the Smad3/4 complex interacts with DNA directly, both TGF-ß and Smad3/Smad4 overexpression induced transcription activation still need the activity of the AP1 complex, at least in the case of the collagenase promoter. While the mechanism by which Smads and AP1 work concertedly to activate transcription remains to be elucidated, these findings are consistent with an earlier observation that Smad2 was found to be associated with another DNA-binding transcription factor FAST1 in the Xenopus system (7).

As described in the last year's Annual Report, we have continued studies on the examination of specific TGf-ß gene responses and growth responses, and the modulation of these effects by the steroid hormones in breast cell lines (outlined in Specific Aim 3). We have established a breast cell culture system to examine the interactions between the negative growth promoting pathway induced by TGF-ß and the positive signaling pathway(s) of the steroid hormones. We have maintained three separate MCF7 breast cancer cell sublines for our study of TGFb growth effects. MCF7 cells are ideal for our studies as they are still responsive to hormones and cytokines, thus representing an early stage of breast cancer. As breast cancers progress, the cells involved typically become resistant to both positive and negative growth factors which makes these later stage cancers immune to standard therapeutic methods. However, in mammary epithelium that is still responsive to growth factors, TGF-ß acts primarily as an inhibitor of cell proliferation. We have identified several MCF7 breast cancer cell lines that show a 90% growth inhibition after TGF-ß addition and a 10-fold proliferation increase after addition of steroid hormones. We have verified that these growth effects are due solely to TGF-ß and estrogen by using the breast cancer cell line, SKBR3, as a negative control. The SKBR3 line represents a late stage cancer since it is known to have lost responsiveness to the signalling pathways of hormones and cytokines. Our cell culture system has revealed a point of interaction between the two opposing signals. When MCF7 cells are exposed to both TGF-ß and estrogen, the growth of the treated cells is intermediate between the two effects alone. The cells are neither fully growth inhibited as if by TGF-ß alone nor are they fully responsive to the growth-promoting signal from estrogen Thus, the presence of estrogen blocks the TGF-ß-growth inhibition, yet TGF-ß blocks the full effect of estrogen-induced proliferation, resulting in a net 5-fold proliferation of the cells.

To determine the mechanism by which these two growth regulators interact, we have initially focused our attention on the cell cycle regulators (a part of this research was reported in the last year's Annual Report). In other cell systems, the down regulation of the G1 cyclin dependent kinases (specifically cdk2 and cdk4) and their cyclin partners (especially cyclin E) contribute to the growth inhibitory effects of TGF-ß. The G1 cyclins and cyclin dependent kinases (cdk's) play an essential role in the progression of a cell through the G1 phase of the cell cycle. Regulation does not occur solely by alterations in the cyclin/cdk expression levels. In recent years, treatment of different types of cells with growth-inhibiting substances such as TGF-ß cause the increased accumulation of factors called cyclin dependent kinase inhibitors (CkI's) which inhibit cyclin/cdk activity. TGF-ß has been shown to suppress the growth of several different cell types by inducing the expression of various cyclin/cdk inhibitors including, p15 (18), p21 (11), and p27 (38,49).

The IGFs have been extensively characterized and have been shown by several groups to be potent mitogens for breast epithelial cells. The importance of the IGFs in breast cancer has been supported by studies in MCF7 cells demonstrating that antibodies against the type I IGF receptor (recognized by both IGFs) block the mitogenic effects of both IGFs (10). Furthermore, type I IGF receptor antibodies reduce the formation of mammary tumors grown in nude mice (1). The stromal fibroblasts are the main source for IGF production in mammary tissue, emphasizing their importance in the development of breast tumors. TGF-ß has been found to induce the expression of insulin-like growth factor binding protein 3 (IGFBP3) in a variety of cell types, including fibroblasts and breast cancer cells (16,20,31). IGFBP3 belongs to a family of secreted proteins that modulate and suppress the growth-stimulatory effects of IGF1 and IGF2 (4,8,36). By binding to the IGFs with high affinity, the IGFBPs play pivotal roles in regulating the availability and bioactivity of the IGFs. IGFBP3 is expressed in most adult tissues, including mammary tissue, and it represents the major circulating IGFBP in adults (13). We intend to test the hypothesis that a stromal/epithelial paracrine loop exists between TGF-ß, IGF1, and IGFBP3.

B. PROGRESS REPORT

In the last twelve months, our work has been focused on two main areas: examination of the functions of Smads, components of the TGF-ß signaling pathway, in the mediation of TGF-ß signal in transactivating downstream target genes; and determination of mechanism by which TGF-ß regulates the growth of breast epithelial cells through the transcriptional activation of the cyclin-dependent kinase inhibitor p15 gene in MCF7 cells as well as the regulation of expression of IGF binding protein, IGFBP3. The progress in these areas are reported below.

B1. Smad3/Smad4 activates the p3TP-Lux reporter by forming a DNA-binding complex.

As discussed in the Introduction, Smad2 and Smad3 have been respectively shown to activate transcription from the PAI-1 promoter, when co-overexpressed with Smad4/DPC4 (26,56). This has led to the hypothesis that Smad2 and Smad3 in combination with Smad4 play specific roles in TGF-ß regulated transcription from these promoter constructs. To examine the molecular nature of Smad-dependent transcriptional activation, we chose to use p3TP-Lux, a luciferase reporter which is a well described and widely used artificial promoter construct empirically designed to have maximal responsiveness to TGF-ß. p3TP-Lux has a 31-nucleotide, AP1-site containing region of the collagenase promoter, concatamerized 5' to an ~400 nucleotide region of the PAI-1 promoter (Fig. 1A). Consistent with previous findings, we observed a transcriptional activation of p3TP-Lux by both Smad3/Smad4 co-overexpression and TGF-ß treatment in CCL64 Mv1Lu cells (Fig. 1B). In contrast, Smad2 fails to activate transcription of p3TP-Lux when co-overexpressed with Smad4. To define the Smad responsive region of p3TP-Lux, we created a reporter construct comprised only of the 31-nucleotide AP1-site containing region concatamerized 5' to a minimal promoter. This 4X WT reporter (Fig. 1A) is not only TGF-ß-responsive, but is also activated in response to Smad3/Smad4 co-overexpression (Fig. 1C). Thus, this 31-nucleotide repeat contains a DNA sequence which is both TGF-ß and Smad responsive.

To determine if Smad3/Smad4 co-overexpression changes the DNA-binding complexes on this 31-nucleotide fragment, we performed electrophoretic mobility shift assays (EMSAs) using a probe consisting of two copies of the 31-nucleotide repeat cut from p3TP-Lux, termed the 2.0 probe (Fig. 2A). When EMSAs were performed using this probe and extracts derived from COS cells co-transfected with epitope tagged Smad3 and Smad4 (Fig. 1D), we observed not only an AP1 containing complex (Complex I), but also a strong additional binding complex (Complex II, Lane 6). Overexpression of Smad3 alone produces a lower level of a complex with similar mobility (Lane 3). Likewise, overexpression of Smad4 produces a complex with similar mobility, as well as a slightly faster migrating complex (Lane 4). In contrast, Smad2/Smad4 co-expression does not produce this complex, but appears similar to Smad4 alone (Lane 5).

One possible explanation for these observations is that Smad3 and Smad4 form a DNA binding complex. Overexpressed Smad3 alone or Smad4 alone could bind DNA with their endogenous Smad partner, whereas co-overexpression would produce a large amount of Smad3/Smad4 binding complex. To test this hypothesis, supershift analysis was performed to determine if HA-tagged Smad3 or Flag-tagged Smad4 are present in the additional binding complex (Complex II). As shown in Figure 1E, both HA and Flag antibodies supershift this complex (Lanes 7, 12 and 13). As expected, a Pan-Fos family member antibody supershifts the faster migrating AP1 complex (Lane 2). This antibody, however, does not shift the Smad3/Smad4 complex (Lane 8), suggesting that although the constitutive binding activity contains a Fos family member, the Smad3/Smad4 complex does not. Finally, the complexes observed with Smad4 overexpression are all Smad4 containing as demonstrated by Flag supershifts (Lane 5). Thus, Smad3 and Smad4, when overexpressed, participate in a DNA binding complex on sequences present in this region of p3TP-Lux.

Recently, the BMP-inducible phosphorylation sites of Smad1 and the TGF-ß-inducible phosphorylation sites of Smad2 have been identified (25,30). Smad3 contains analogous sites of phosphorylation at its C-terminus and has recently been shown to be phosphorylated at those sites in response to TGF-ß (H. Lodish, personal communication). Based on this information, we created a phosphorylation deficient mutant of Smad3, Smad3MT, and assayed its ability to participate with Smad4 in a DNA binding complex. Although the expression levels were similar to wild-type Smad3, Smad3MT was unable to form a DNA binding complex with Smad4 (Fig. 1D, Lane 7). The results with this mutant suggest that an intact carboxyl-terminus of Smad3 is essential for formation of the DNA binding complex. This mutation possibly interferes with the ability of Smad3 to form a heteromeric complex with Smad4 and thus precludes formation of the DNA-binding complex.

B2. Smad3/Smad4 complex binds to a bipartite site through the DNA-binding activity of Smad4

To more precisely determine the DNA sequences to which the Smad3/Smad4-containing complex binds, we systematically mutated the 2.0 probe (Fig. 2A). As expected, mutation of the AP1 binding sites eliminated the Fos-containing shifted complex. The Smad3/Smad4 complex, however, was still present on the AP1 site mutant probe, although in somewhat decreased amounts (Fig. 2B, Lane 3). This further suggests that the Smad3/Smad4 complex is not binding through AP1. We next designed three separate scanning mutants to encompass the entire 2.0 probe in search of the specific sequence which confers Smad3/Smad4 binding (Fig 2A). As shown in Figure 2B, scanning mutant #1 eliminates both the AP1 and the Smad3/Smad4 complexes, while scanning mutant #2 specifically eliminates the Smad3/Smad4 complex leaving the AP1 complex intact. Scanning mutant #3 has no effect on the binding of either complex. Thus, the region necessary for Smad3/4 complex binding lies within the bases mutated in scanning mutants #1 and #2.

Methylation interference was then used to more precisely define which guanine residues within the 2.0 probe are contacted by the Smad3/Smad4 complex. The results confirm the mutagenesis results in that there is a single protected guanine residue that is located within the region predicted by the scanning mutagenesis (data not shown). Both sites of this two site probe have almost completely protected guanine residues. This suggests that both sites are being contacted in this single Smad3/Smad4 complex. Mutation of 6 nucleotides surrounding this protected guanine (GACACC) in either the 5' or 3' site of the 2.0 probe was sufficient to eliminate Smad3/Smad4 binding (data not shown), further indicating the requirement of a bipartite site for Smad3/Smad4 complex formation. In addition, a probe containing only one of these 31-nucleotide repeats (one half of the probe used in these experiments) was completely unable to bind the Smad3/Smad4 complex in EMSA assays (data not shown).

We next sought to determine if either Smad3 or Smad4 themselves were directly binding this DNA sequence. To determine this, we generated GST fusions of both proteins and used these purified reagents in EMSA with the 2.0 probe. Although Smad3 is incapable of binding, GST-Smad4 directly binds the 2.0 wild type and AP1 mutant probes, but does not bind the 2.0 Smad binding site mutant probe (Fig. 3). Thus, the complex seen on Smad3/Smad4 co-overexpression may be the result of a direct DNA interaction by Smad4. Although Smad4 binds directly to this DNA sequence, Smad3 may modulate its binding affinity or affect its binding specificity. An altered specificity of Smad4 in complex with different Smads may explain why Smad2/Smad4 complexes do not bind this sequence, but Smad3Smad/4 complexes do. The ability of Smad4 to directly bind this DNA sequence also explains the additional shifted complex observed when Smad4 is overexpressed alone in COS cells (Fig. 1D, E, Lane 4). That complex is most likely Smad4 bound without endogenous Smad3, which is further supported by the ability of the Flag antibody to shift this complex (Fig. 1E, Lane 5).

B3. Smad3/Smad4 induced transcriptional activation requires the presence of AP1 sites

All experiments described to this point have been performed in the context of Smad over-expression. We next looked in vivo for a Smad containing DNA-binding complex. Ideally, such a complex would be predicted to be TGF-ß-inducible and phosphorylation dependent. EMSAs were performed using the 2.0 probe and nuclear extracts prepared from either TGF-ß treated or untreated Mv1Lu cells. In the absence of TGF-ß treatment, Mv1Lu cells contain a constitutive Fos-containing binding complex similar to the Fos complex in COS cells (Fig. 4C, Lane 2). Upon TGF-ß treatment, a slower migrating complex appears within 5 min, peaks in 15 min and disappears after 4 hrs. (Fig. 4A). This time course parallels the TGF-ß-dependent phosphorylation kinetics of endogenous Smad proteins (55). The inducibly bound complex is sensitive to phosphatase treatment, suggesting that its binding is phosphorylation dependent (Fig. 4B). Finally, the presence of Smad4 in this TGF-ß inducible complex was confirmed by supershift analysis with a Smad4-specific antibody (Fig. 4C, Lane 5). Unfortunately, our Pan-Smad antibodies which recognize Smad1, 2, 3 and 5 could not supershift either the endogenous Smad4-containing complex nor the Smad3/4 overexpressed complex from COS cells because of their relatively low affinity for Smad3 (data not shown). Therefore, we cannot unequivocally show that Smad3 is a component of the TGF-ß-inducible shifted complex in Mv1Lu cells. However, the inducible complex comigrates with the Smad3Smad/4 complex from COS lysates (data not shown) and shares an identical binding site within the 2.0 probe as revealed by gel shift analysis using the panel of 2.0 probe mutants (data not shown). These data combined with the fact that no other Smad in combination with Smad4 from COS lysates is able to bind the 2.0 probe, provides strong evidence that the inducible complex in Mv1Lu cells contains Smad3 and Smad4.

Having identified the specific region of the 2.0 probe which was capable of conferring Smad3/Smad4 binding, we examined the functional consequences of Smad binding site and AP-1 site mutations in the context of the 4X WT reporter in Mv1Lu cells. The results indicate that the AP1 sites are critically important for induction by both TGF-ß and Smad3/Smad4 co-overexpression (data not shown). Surprisingly, mutation of the Smad binding site had no effect on induction by TGF-ß or by Smad3/Smad4 co-overexpression.

The apparent dispensability of the Smad binding site within this reporter could be explained in several ways. Smad complex binding may have effects which are not assayed in these transient transfection experiments. If, for example, Smad binding plays a role in the recruitment of other transcription factors to adjacent sites (e.g. AP1), or in re-arrangement of chromosome structure to provide accessibility of other transcription factors to their binding sites, an effect in a transient transfection assay may be difficult to observe. The transient nature of Smad binding would be consistent with this type of role in transcriptional activation. Alternatively, in the context of our promoter constructs, Smad binding may not be required, but in other promoter contexts, Smad binding may be essential. Although the functional consequences of Smad binding remains uncertain, we have clearly demonstrated that Smad3/Smad4 co-overexpression can activate transcription through AP1 binding sites. This raises the possibility that the Smads have at least two separable functions. One is a direct effect through its sequence specific DNA binding. The second is a potentially more indirect effect to activate AP1-mediated transcription, perhaps through interactions with specific TAFs, and may explain the widely observed phenomenon that TGF-ß can activate transcription through AP1 binding sites.

B4. The PAI-1 promoter contains binding sites for Smad3/Smad4

Our finding that overexpressed Smads can bind to DNA in a sequence specific fashion prompted us to determine if the promoters of other potentially Smad regulated genes contain similar sequences. Naturally, we chose the PAI-1 promoter as a part of it is contained in the p3TP-Lux

reporter construct and the promoter per se is shown to be activated by overexpression of both Smad2/Smad4 and Smad3/Smad4 (26,56). By using the same approach as described above, we have found a region of the PAI-1 promoter to contain a Smad binding site which is similar to the one found in p3TP-Lux. As shown in Fig. 5A, the result of EMSA using COS cell lysates demonstrates that a complex consisting of Smad3/Smad4 is formed upon the probe derived from the PAI-1 promoter (Fig. 5B). We are currently in the process of performing an in vitro footprinting experiment, using bacterially produced Smad3 and Smad4 proteins, to map precisely the binding sequences by following a standard procedure. These in vitro binding assays have been aided by our findings that Smads appear to have a high tendency to form homomeric and heteromeric complexes when they are overexpressed in COS cells or generated as fusion proteins in bacterial and mixed together in test tubes, which is consistent with the recently published results of Smads interactions in the yeast system (54). Consequently, many of the in vitro experiments in the proposal will take advantage of this feature of Smad proteins by using COS cell lysates or GST-fusion proteins.

Meanwhile, we are conducting a scanning mutagenesis of this promoter region, combined with a functional analysis of the Smad binding sites by testing the TGF-ß and Smad3/Smad4 responsiveness of various mutants of the PAI-1 promoter in the same way as was done for the p3TP-Lux reporter construct in Mv1Lu and human keratinocyte HaCaT cells. The immediate goal is to define precisely the DNA sequences recognized by the Smad3/Smad4 complex and determine if the binding site plays a role in mediating the transactivating signal of TGF-ß/Smads in the transient transfection functional assay.

As discussed above, the activity of AP1 is required for the TGF-ß/Smads mediated transactivation in p3TP-Lux. There is one putative AP1 consensus site identified previously in this 400 bp region of the PAI-1 promoter (22), but whether it functions as a true AP1 complex binding site remains to be determined. We will perform the same type of experiments for the identification of Smad3/Smad4 binding sites to identify the AP1 sites responsible for the mediation of TGF-ß/Smads transactivating signal.

B5. Create a Smad3 homozygous null mouse through targeted gene disruption.

Most of our experiments intended to elucidate the molecular mechanism of Smad/TGF-ß-mediated signal transduction have been done in tissue culture cells. However, these experiments may not reveal some of the potential physiological roles of Smads in vivo, particularly the involvement of Smads as tumor suppressors in the process of carcinogenesis. To fully understand the roles of Smads in the context of a whole organism, we have already begun the process of generating a Smad3 homozygous null mouse through targeted gene disruption using the service provided by the Transgenic Facility at Duke Cancer Center. Once the animals are generated, the interpretation of phynotypic changes in those KO animals, including morphological, physiological, and pathological abnormalities, will in most cases require a basic understanding of the potential mechanisms behind those changes. This is often achieved by conducting biochemical and biological assays using primary fibroblasts and thymocytes under in vitro culture conditions. The obvious advantage of such assays is the use of cells containing defined genetic alterations, such as the absence of Smad3, in comparison to the use of established immortal tissue culture cell lines which often carry multiple and mostly unknown genetic lesions. It will be crucial to correlate any changes detected in such in vitro assays with the phynotypic changes observed in animals so that a meaningful mechanistic analysis and determination can be carried out.

To create the targeting vector, the first 300 nucleotides of the human Smad3 coding sequence was used as a probe to screen a mouse (strain 129) genomic library. Several positive genomic clones were isolated. One clone was found to contain 14 Kb of genomic sequence including the first exon of Smad3 gene. The first exon contains the initial sixty-nine amino acids of Smad3, composing part of the conserved MH1 domain (33). In this coding region of the Smad3

gene, the mouse and human sequences are identical on the amino acid level, suggesting an important function for this portion of the protein. Following this first exon is a large intron of at least 7 Kb. This genomic clone also contains approximately 6 Kb of sequences 5' to the Smad3 first exon, representing the Smad3 promoter sequences. In order to create a targeting construct which could completely eliminate Smad3 expression when it is inserted into the mouse genome through homologous recombination, a targeting vector was designed in which all of the coding sequence in the first exon of Smad3 gene as well as part of the first intron were removed. This sequence is replaced by a neomycin expression cassette under the control of the PGK promoter for positive selection. The total homologous sequence in this targeting vector is approximately 6.5 Kb, and is flanked by a thymidine kinase expression cassette for negative selection. This targeting strategy does not remove any sequences of the 5' promoter region of Smad3. Incorporation into the endogenous Smad3 locus can be analysed both by PCR through the 1.1 Kb short arm of the targeting vector, and by genomic Southern blotting.

At this point, the targeting vector shown in Fig. 7 has been electroporated into the mouse ES cell line and several positive ES cell clones harboring the disrupted Smad3 allele have been identified by PCR. These PCR positive ES cells will be re-screened by Southern blot analysis and those are properly targeted, as verified by Southern blot, and are karyotypically normal will be injected into blastocysts to utimately produce chimeric mice. These mice will then be screened for germline transmition of the disrupted allele, to produce heterozygous and ultimately homozygous Smad3 null mice following standard procedures.

B6. TGF-ß signaling and cell cycle progression in MCF7 cells.

We have previously demonstrated that TGF-ß induces the expression of the cyclin-dependent kinase inhibitors, p15 and p21, and induction of these proteins is correlated with TGF-ß-induced growth inhibition in human keratinocyte HaCaT cells (11,18). Subsequently, we have found that p15, but not p21, appears to be a major component of TGF-ß-mediated growth inhibition in MCF7 cells. As reported last year, the levels of p15 mRNA increase approximately 10-fold in as little as 7 hours after TGF-ß addition, whereas the presence of estrogen does not increase p15 mRNA levels. However, when MCF7 cells are exposed to both estrogen and TGF-ß, the positive growth signals initiated by estrogen block TGF-ß's induction of p15 mRNA. This block in p15 mRNA induction correlates with the growth effects observed in MCF7 cells after TGF-ß and estrogen addition. Thus, p15 represents an intersection of both pathways through which the growth of the breast epithelial cells may be manipulated. Interestingly, we have also found that other mitogenic substances including the insulin-like growth factors (IGF1 and IGF2) block p15 mRNA induction by TGF-ß in a similar manner, supporting their roles as common mitogenic growth factors. To investigate whether estrogen and the IGFs affect TGF-ß-induced p15 expression through a direct or indirect mechanism, we tested if the p15 promoter construct containing 751 basepairs of the promoter region, which was shown to be responsive to TGF-ß in HaCaT cells (28), could be activated by the TGF-ß signal in MCF7 cells. To our surprise, the promoter construct was not induced by TGF-ß, whereas the positive control of the PAI-1 promoter construct was induced by 5 to 10 folds. To investigate if the stability of p15 mRNA changes in respond to TGF-ß, we treated the MCF7 cells with or without TGF-ß in the presence of 10 ug/ml actinomycin D, an inhibitor of RNA synthesis, and analyzed the mRNAs with the RNase protection assay. The results indicate that there is no change in the half life of p15 mRNA in cells treated or untreated with TGF-ß, suggesting that the 10-fold increase in p15 mRNA steady-state level in response to TGF-ß is primarily the result of transcriptional activation. Therefore, it is likely that the p15 promoter construct used in the assay may not contain the TGF-ß responsive elements functional in MCF7 cells. This assessment is consistent with our previous observation that the same promoter construct failed to show induction by TGF-ß in Mv1Lu cells in which the p15 mRNA was also seen to be strongly induced by TGF-ß. Based on these results, we have isolated additional genomic DNA sequences representing a larger region of the p15 promoter and currently in the process of testing if the region contains TGF-ß responsive elements which can mediate the

TGF-ß effect in MCF7 cells. If novel TGF-ß responsive elements other than the previously defined binding site for Sp1 transcription factors are identified in the p15 promoter, we will study the mechanism by which the p15 gene is regulated by TGF-ß and estrogen in MCF7 cells.

B7. TGF-ß mediated regulation of IGFBP3 expression.

As reported last year, we have also investigated an additional aspect of TGF-ß-induced growth inhibition in the breast cancer epithelial cells. The induction of p15 in the MCF7 cells is an example of TGF-ß's direct effect on the cell cycle machinery in order to slow the growth of the breast cancer epithelial cells. In addition to these effects, TGF-ß appears to have a broader effect on breast cell populations through paracrine actions. While breast cancers almost exclusively originate in epithelial cells such as the MCF7 cells, they cannot develop significantly without the surrounding stromal components. The interactions between the stromal fibroblasts and the mammary epithelial are particularly important in both neoplastic and normal development of mammary tissue (for review, see (10). Involved in these stromal/epithelial interactions are the growth promoting factors such as the steroid hormones and the insulin-like growth factors (IGFs), and the growth inhibiting factors such as the TGF-ß's. We have expanded our studies of TGFß-induced growth inhibition in the breast cancer by examining TGF-ß's role in the stromal/epithelial paracrine loop. Specifically, we have begun to focus on the roles of TGF-ß and IGF1 in the progression of breast cancer.

As reported last year, we have established a collaboration with Dr. John Fowlkes from Department of Pediatric Endocrinology at University of Kentuky to test this potential paracrine role of TGFb in breast cancer cells. A fetal lung fibroblast cell line, MRC9, secretes insulin-like binding protein 3 (IGFBP3) in the presence of TGF-ß. MRC9 cells are not growth inhibited by TGF-ß, yet TGF-ß induces a 20-fold increase in both IGFBP3 mRNA and protein in these cells (see last year's report). To study the mechanism of TGF-ß induced expression of IGFBP3, we transfected a reporter construct containing 1.8 Kb of the IGFBP3 promoter (9) into MRC9 cells and measured the luciferase activity after TGF-ß treatment. The results were disappointing as no induction of the promoter was observed in the presence of 100 pM TGF-ß1. To rule out that the increase in the steady-state level of IGFBP3 mRNA in response to TGF-ß is due to an increase in RNA stability, we measured the half life of IGFBP3 mRNA in the presence or absence of TGF-ß. The results indicate that the increased expression of IGFBP3 is primarily due to transcriptional activation of the IGFBP3 gene and the TGF-ß responsive elements are likely outside the 1.8 Kb promoter region of the gene. In fact, another study have found the p53-responsive elements of the IGFBP3 gene are located in the first intron rather than the 5'-region of the transcriptional start site of the gene (5). We have already begun the process of identifying the potential TGF-ß regulatory elements within the IGFBP3 gene by isolating genomic clones containing both the first intron and a larger piece of the 5'-promoter region. If the TGF-ß responsive elements are found, we will subsequently study the mechanism of TGF-ß mediated induction of the IGFBP3 gene and ultimately investigate the mechanisms of TGF-ß growth inhibition of breast epithelial cells in a novel cell culture model of the stromal fibroblast/epithelial interactions observed in clinical breast cancer cases.

C. CONCLUSIONS

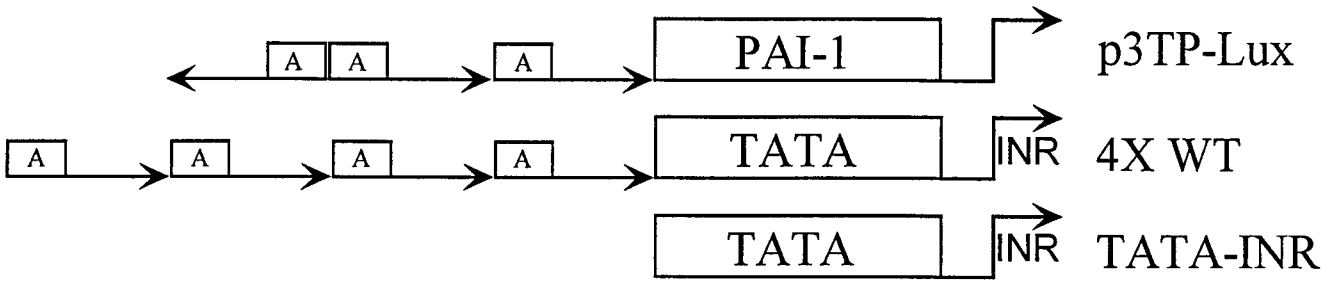
Significant progress has been made in advancing the goal described in the Specific Aim 4 of the original proposal. As discussed in the section of Introduction, available evidence strongly suggest that Smads play a more global role as regulators of multiple biological responses to ligands of the TGF-ß superfamily by participating in transcriptional activation of multiple target genes. Specifically for TGF-ß, Smads may act as intermediates for a number of TGF-ß signaling pathways leading to cell growth regulation and extracellular matrix deposition. Loss of function mutations in the Smads could disrupt those important TGF-ß signaling pathways and contribute to tumorigenesis in multiple types of tissues and organs, including the breast, in humans. Inside the nucleus, Smads may work to modulate transcription by binding to specific DNA elements in promoters of the target genes and interacting with a spectrum of different DNA-binding transcription factors or factors of the basal transcription machinery. As described in the last section, our recent progress in determining the biochemical properties of Smads in mediating the biological effects of TGF-ß, with the collagenase and PAI-1 gene promoters as model systems, has provided us with an opportunity for studying this novel TGF-ß signaling mechanism, a key step toward an understanding of how this multifunctional hormone regulates so many cellular functions. A comprehensive analysis of the role of Smads in modulating the expression of endogenous PAI-1 gene, combined with a determination of the identities and physiological roles of additional genes which are potentially under the control of this signaling pathway, will lead to further elucidation of the program of TGF-ß signal transduction. The exploration of the physiological role of Smad3 by the strategy of genetic manipulation of the Smad3 gene will address the question whether Smad3, like Smad2 and Smad4, function as a tumor suppressor in animals.

As described in the second part of the report, cell growth is dictated by a delicate balance between positive and negative extracellular proliferative signals. Loss of this balance is a key factor leading to cancer. The goal of this part of research (Specific Aim 5 in the original proposal) aims to determine the role of the antagonistic relationship between two opposing growth signals, IGF and TGF-ß, in mammary tumorigenesis. These two factors represent opposing forces which contribute to the balance of proliferation and growth inhibition of normal mammary growth and development of normal mammary growth and development. As discussed above, TGF-ß acts to directly affect cell cycle progression and cause a G1 cell cycle arrest by activating several cyclin dependent kinase inhibitors, including p15, p21, and p27. In addition to these effects, TGF-ß has a broader effect on cell populations through paracrine actions. It has been recently shown that TGF-ß can induce the expression of IGFBP-3, a protein which inhibits the action of the mitogenic IGFs. We have started to test this hypothesis by establishing a model system in which the effects of TGF-ß through IGFBP-3 can be studied. We intend to subsequently define the molecular mechanisms by which TGF-ß induces IGFBP-3 by identifying TGF-ß regulatory elements in the IGFBP-3 promoter and the corresponding cellular components acting at these promoter elements.

Consequently, the results of these studies will represent a major step toward the elucidation of a novel TGF-ß signaling pathway downstream of the receptor complex. In addition, an elucidation of the precise signaling mechanism by which TGF-ß exerts its biological effects will contribute greatly to our general knowledge of how other members of the TGF-ß superfamily of hormones function. It has been suggested that cellular transformation and tumorigenesis in vivo are sequential multistep processes and loss of cellular responsiveness to negative growth signals, such as TGF-ß, may represent a crucial step during those processes. Furthermore, since the immune system plays a vital role in controlling tumor growth, expansion, and metastasis at the organismal level, disregulation in immune functions as a result of imbalances in the activities of cytokines, such as TGF-ß, could have profound effect on the development of cancers in humans. Therefore, results from the outlined experiments in this proposal will not only reveal the mechanism of the TGF-ß signal transduction pathway, but also provide insight into molecular events that lead to carcinogenesis and other human diseases.

Figure 1

A


A
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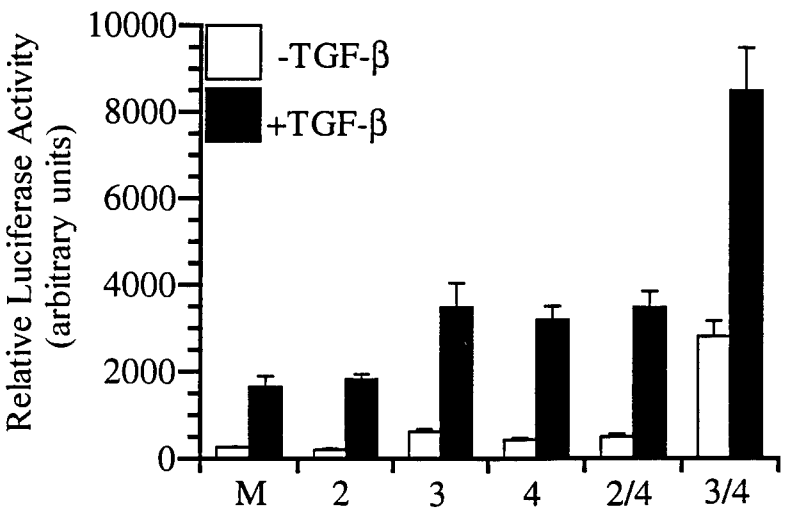
TATA-INR

B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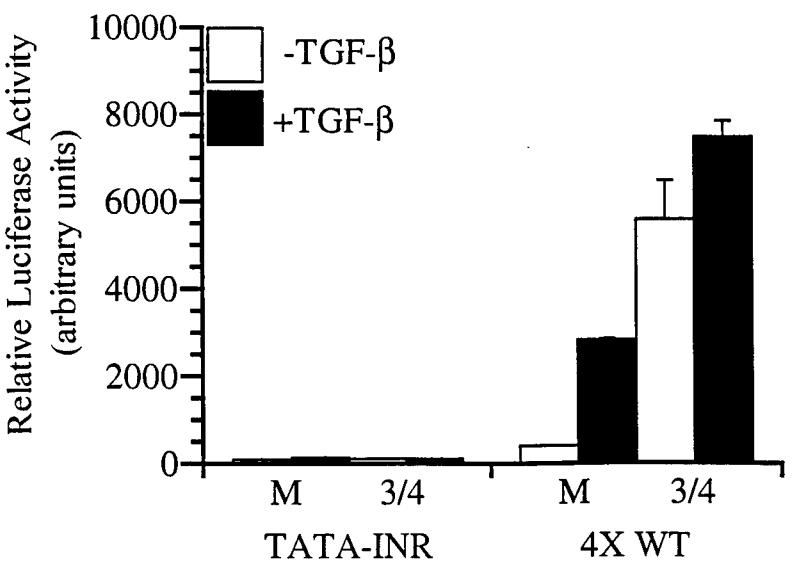
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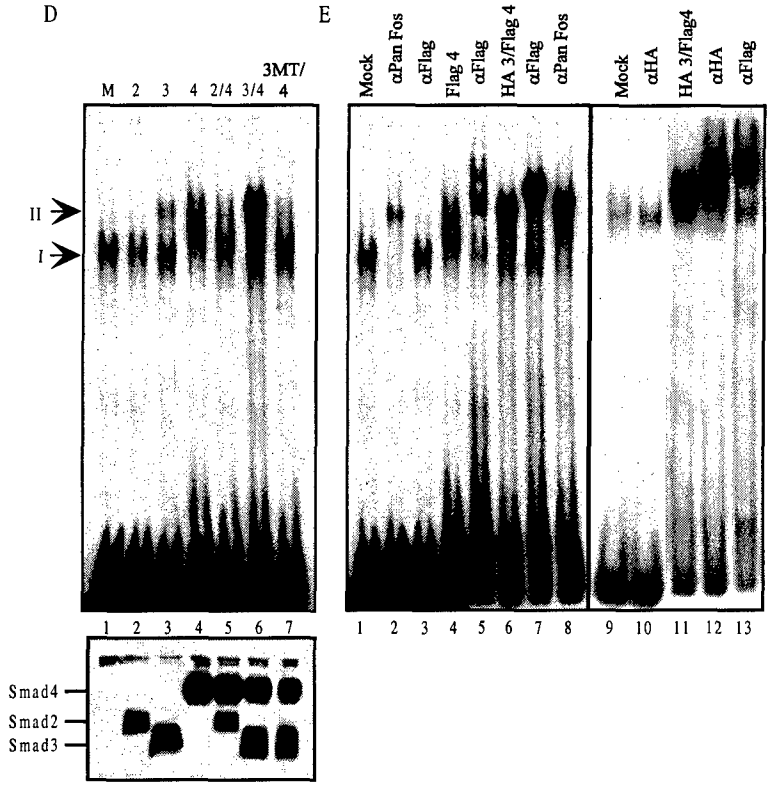
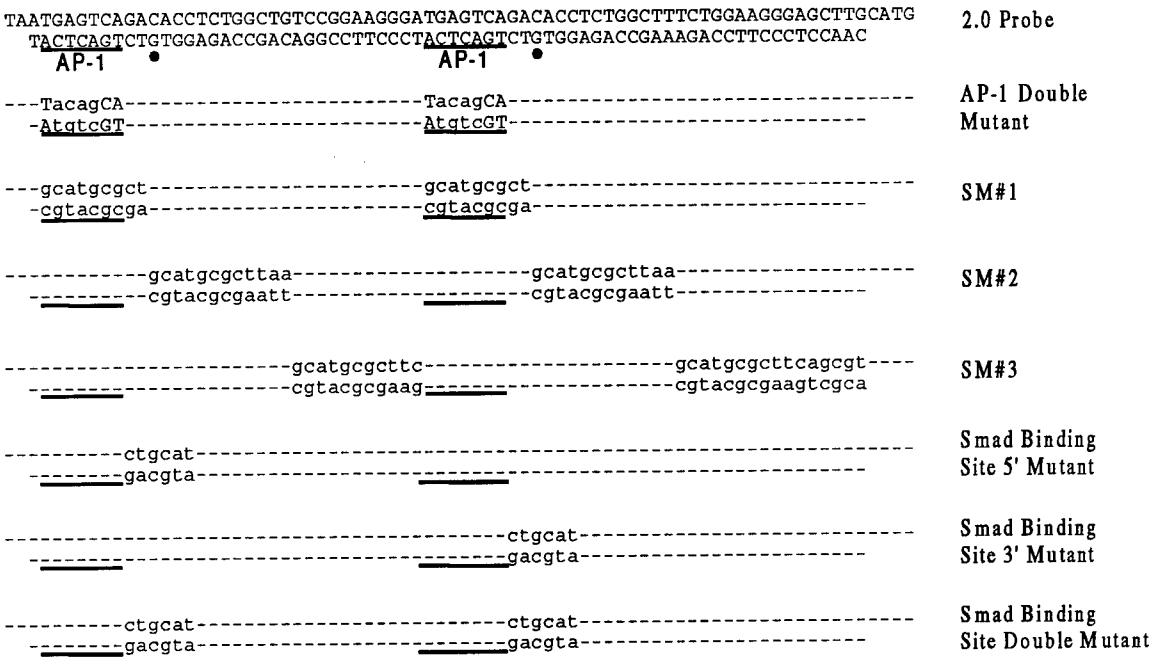


Figure 1. COS cells were transfected with Smad cDNAs as indicated. Whole cell lysates were prepared and subjected to gel shift analysis using the 2.0 probe as described in Fig.2A. The inset below panel D shows the relative expression of Smad2, 3, and 4 in the lysates. d) Gel shift with Smad lysates. 3MT/4 is the phosphorylation deficient mutant of Smad3 described in the text. e) Gel supershift analysis with αPan Fos (Santa Cruz, K25), αHA (BABCO) or αFlag (Kodak, IBI) antibodies.

A



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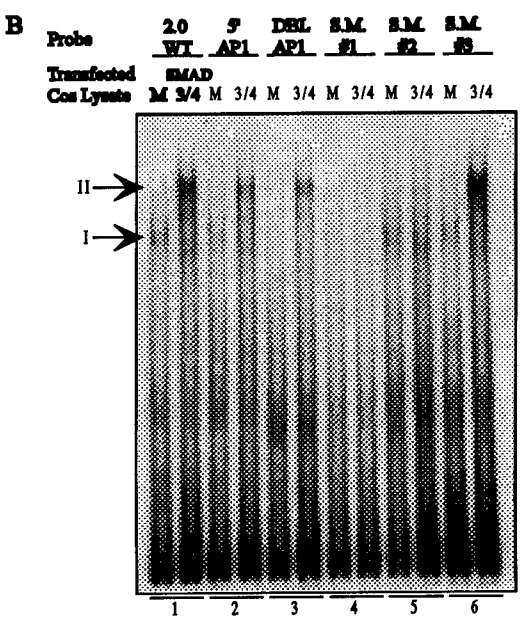


Figure 2. Mock COS lysates (M) or overexpressing Smad3/4 (3/4) were used in gel shift analysis with the probes described in (A). Complex I is the endogenous AP1 complex. Complex II is Smad3/4 bound to the DNA probe.

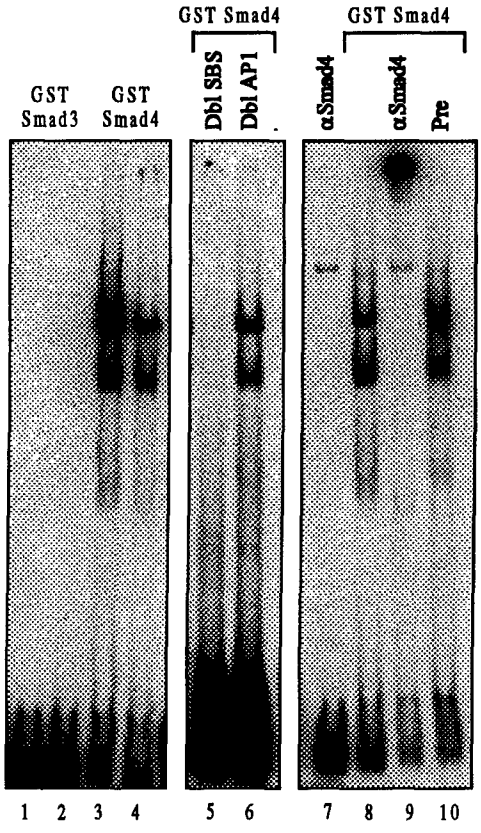
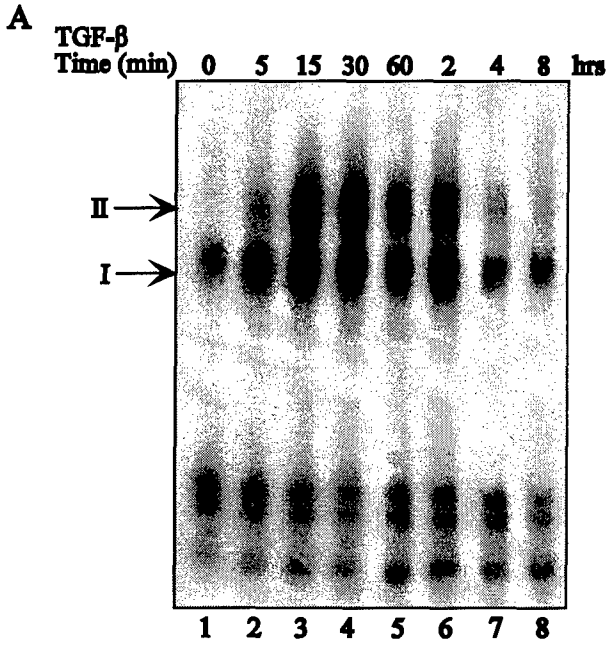
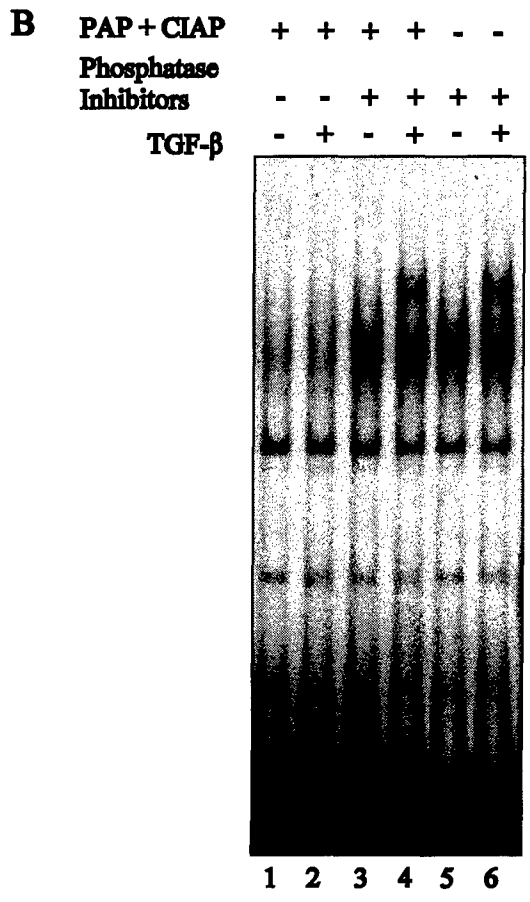


Figure 3. GST Smad3 or GST Smad4 were purified on GST beads and eluted for use in gel shift analysis with the 2.0 probe as in Fig.2 (Lanes 1-4). Mutant probes were used to show binding specificity (Lanes 5,6) and the Smad4 antibody shifts the complex that forms in the presence of GST Smad4 (Lane 9). Pre immune serum fails to shift the complex (Lane 10).





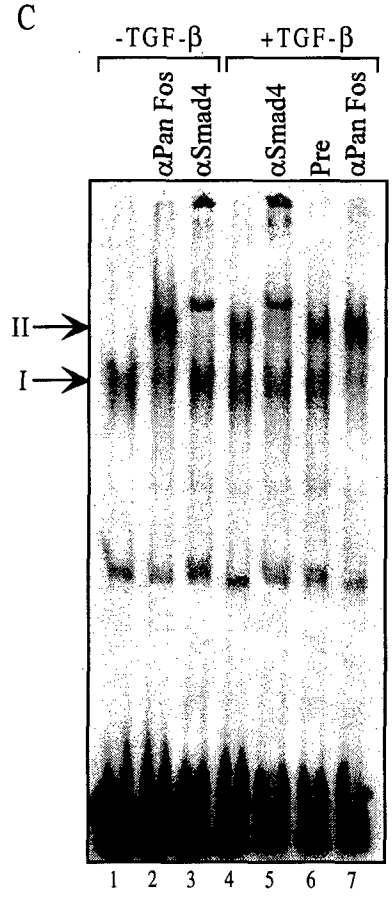
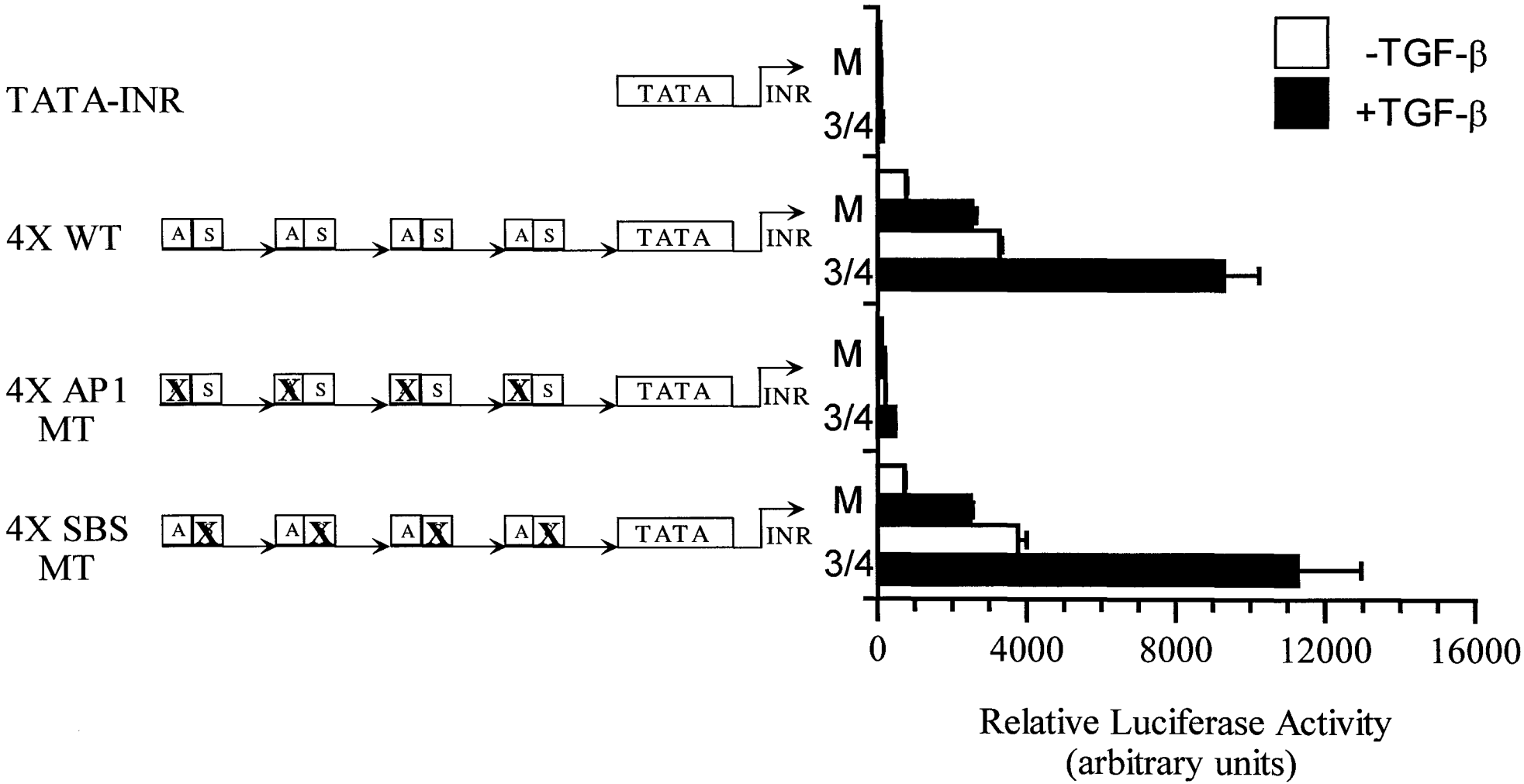


Figure 4. a) Mv1Lu cells were treated with 100 pM TGF-β1 for the indicated lengths of time before whole cell lysates were produced for gel shift analysis with the 2.0 probe as in Fig.2. b) The TGF-β inducible complex (II) is sensitive to phosphatase (Lane 2). c) Antibody supershift analysis confirms the presence of Smad4 in the endogenous complex (Lane 5).

Figure 5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4X SBS MT
A X
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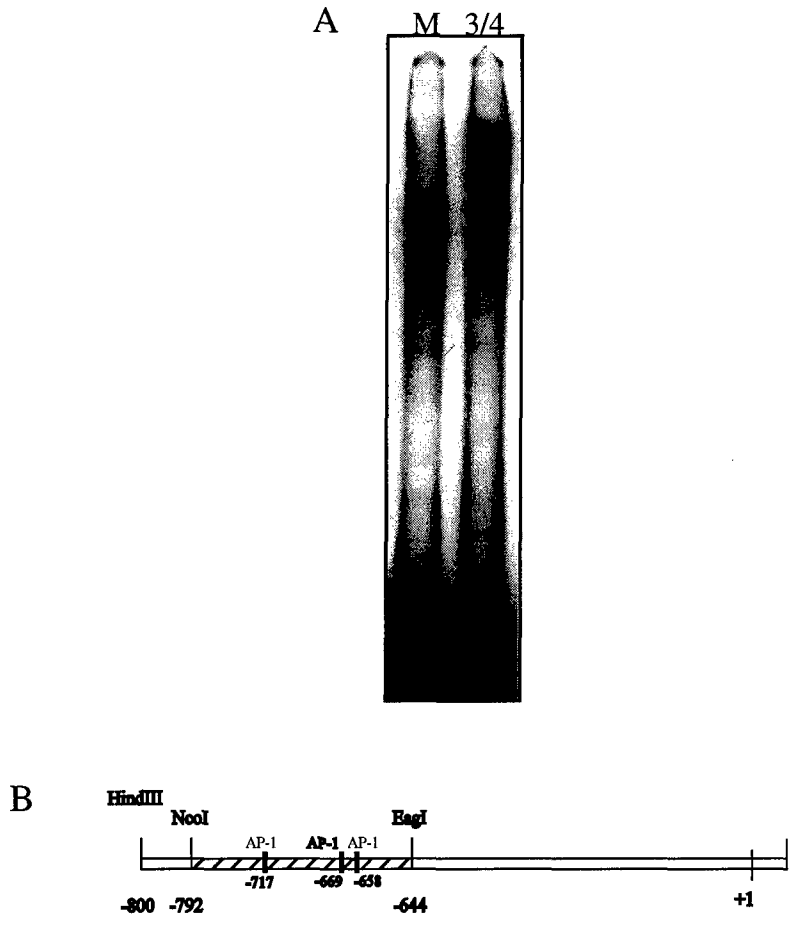
Relative Luciferase Activity
(arbitrary units)



PAI-1 promoter region

Figure 6. (a) A restriction fragment from the PAI-1 promoter, as diagrammed in (b), was radiolabelled and used in a gel shift assay with extracts from COS cells either mock transfected, M, or cotransfected with expression constructs for Smad3 and Smad4, 3/4. A DNA binding complex composed of endogenous proteins is present in the mock transfected extracts. An additional, slower migrating complex is formed upon overexpression of Smad3 with Smad4. (b) Schematic diagram representing the region of the PAI-1 promoter shown in (a) to bind the Smad3/Smad4 complex.

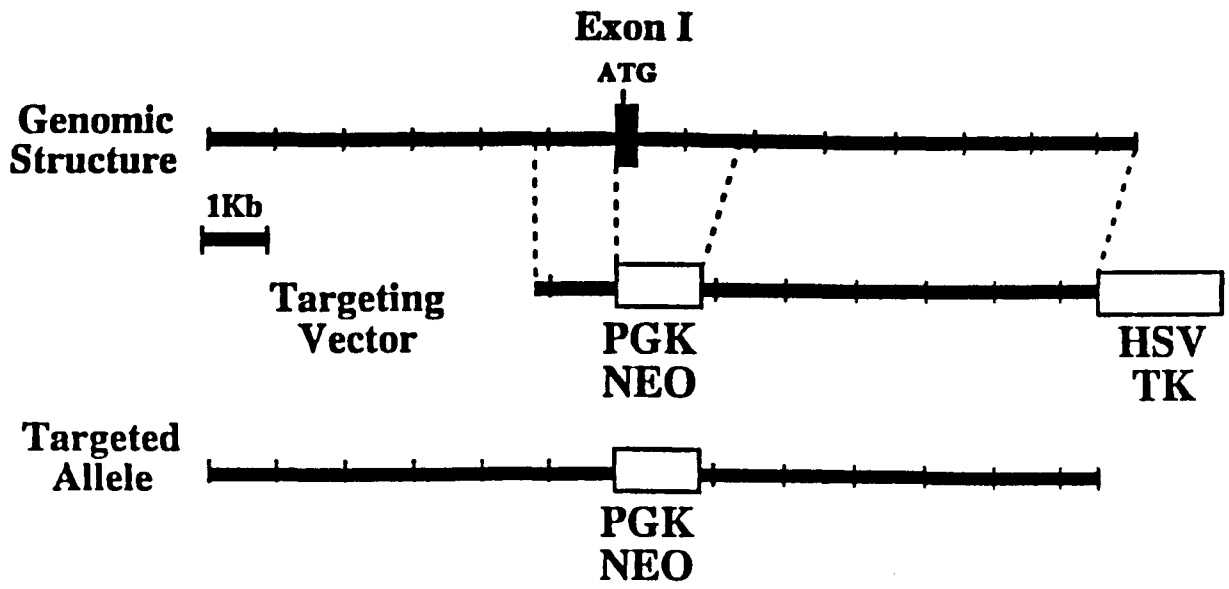


Fig. 7. Partial genomic structure of Smad3 gene and targeted allele.

E. REFERENCES

1. Artega, C. L., L. Kitten, E. Coronado, S. Jacobs, F. C. Kull, D. C. Allred, and C. K. Osborne. 1989. Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. *J. Clin. Invest.* **84**:1418-1423.

2. Baker, J. and R. Harland. 1997. A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. *Genes Devel.* **10**:1880-1889.

3. Barrett, M. T., M. Schutte, S. E. Kern, and B. J. Reid. 1996. Alletlic loss and mutational analysis of the DPC4 gene in esophageal adenocarcinoma. *Cancer Res.* **56**:4351-4353.

4. Baxter, R. C. and J. L. Martin. 1989. Binding proteins for the insulin-like growth factors: structure, regulation, and function. *Prog. Growth Factor Res.* **1**:49-68.

5. Buckbinder, L., R. Talbott, S. Velasco-Migel, I. Takenaka, B. Faha, B. R. Seizinger, and N. Kley. 1995. Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* **377**:646-649.

6. Chen, R. -H., P. J. Miettinen, E. M. Maruoka, L. Choy, and R. Derynck. 1995. A WD-domain protein that is associated with and phosphorylated by the type II TGF-beta receptor. *Nature* **377**:548-552.

7. Chen, X., M. J. Rubock, and M. Whitman. 1996. A transcriptional partner for MAD proteins in TGF-beta signaling. *Nature* **383**:691-696.

8. Conover, C. A., J. T. Clarkson, S. K. Durham, and L. K. Bale. 1991. Cellular actions of insulin-like growth factor binding protein-3, p. 255-266. In Current Directions in Insulin-like Growth Factor Research. CRC Press,

9. Cubbage, M. L., A. Suwanichkul, and D. R. Powell. 1990. Insulin-like growth factor binding protein-3: Organization of the human chromosomal gene and demonstration of promoter activity. *J. Biol. Chem.* **265**:12642-12649.

10. Cullen, K. J. and M. E. Lippman. 1992. Stromal-epithelial interactions in breast cancer *Cancer Treat. Res.* **61**:413-431.

11. Datto, M. B., Y. Li, J. Panus, D. J. Howe, Y. Xiong, and X-Y. Wang. 1995. TGF-beta mediated growth inhibition is associated with induction of the cyclin-dependent kinase inhibitor, p21. *Proc. Natl. Acad. Sci.* **USA** 92:5545-5549.

12. Derynck, R., W. M. Gelbart, R. M. Harland, C. -H. Heldin, S. E. Kern, J. Massague, D. A. Melton, M. Mlodzik, R. W. Padgett, A. B. Roberts, J. Smith, G. H. Thomsen, B. Vogelstein, and X. -F. Wang. 1996. Nomenclature: Vertebrate mediators of TGFbeta family signals. *Cell* **87**:173.

13. Ellis, M. J. C., C. Singer, A. Hornby, A. Rasmussen, and K. J. Cullen. 1994. Insulin-like growth factor mediated stromal-epithelial interactions in human breast cancer. *Br. Can. Res. Treat.* **31**:249-261.

14. Eppert, K., S. W. Scherer, H. Ozcelik, R. Pirone, P. Hoodless, H. Kim, L. -C. Tsui, B. Bapat, S. Gallinger, I. L. Andrulis, G. H. Thomsen, J. L. Wrana, and L. Attisano. 1996. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86**:543-552.

15. Graff, J. M., A. Bansal, and D. A. Melton. 1996. Xenopus Mad proteins transduce distinct subsets of signals for the TGFbeta superfamily. *Cell* **85**:479-488.

16. Gucev, Z. S., Y. Oh, K. M. Kelley, and R. G. Rosenfeld. 1996. Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor beta2-induced growth inhibition in human breast cancer cells. *Cancer Res.* **56**:1545-1550.

17. Hahn, S. A., M. Schutte, A. T. M. S. Hoque, C. A. Moskaluk, L. T. da Costa, E. Rozenblum, C. L. Weinstein, A. Fischer, C. J. Yeo, R. H. Hruban, and S. E. Kern. 1996. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271**:350-353.

18. Hannon, G. J. and D. Beach. 1994. p15INK4B is a potential effector of TGF-B-induced cell cycle arrest. *Nature* **371**:257-261.

19. Hoodless, R. A., T. Haerry, S. Abdollah, M. Stapleton, M. B. O'Connor, L. Attisano, and J. L. Wrana. 1996. MADR1, a MAD-related protein that functions in BMP2 signaling pathways. *Cell* **85**:489-500.
20. Huynh, H., X. Yang, and M. Pollak. 1996. Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. *J. Biol. Chem.* **271**:1016-1021.
21. Kawabata, M., T. Imamura, K. Miyazono, M. E. Engel, and H. L. Moses. 1995. Interaction of the transforming growth factor-beta type I receptor with farnesyl-protein transferase-α. *J. Biol. Chem.* **270**:29628-29631.
22. Keeton, M. R., S. A. Curriden, A. -J. van Zonneveld, and D. J. Loskutoff. 1991. Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor beta. *J. Biol. Chem.* **266**:23048-23052.
23. Kim, S. K., Y. Fan, V. Papadimitrakopoulou, G. Clayman, W. N. Hittelman, W. Hong, R. Lotan, and L. Mao. 1996. DPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. *Cancer Res.* **56**:2519-2521.
24. Kingsley, D. M. 1994. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Devel.* **8**:133-146.
25. Kretzschmar, M., F. Liu, A. Hata, J. Doody, and J. Massague. 1997. The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes Devel.* **11**:984-995.
26. Lagna, G., A. Hata, A. Hemmati-Brivanlou, and J. Massague. 1996. Partnership between DPC4 and SMAD proteins in TGF-beta signaling pathways. *Nature* **383**:832-836.
27. Lechleider, R. J., M. P. de Caestecker, A. Dehejia, M. H. Polymeropoulos, and A. B. Roberts. 1996. Serine phosphorylation, chromosomal localization, and transforming growth factor-beta signal transduction by human bsp-1. *J. Biol. Chem.* **271**:17617-17620.
28. Li, J. -M., M. A. Nichols, S. Chandrasekharan, Y. Xiong, and X. -F. Wang. 1995. Transforming growth factor β activates the promoter of cyclin-dependent kinase inhibitor p15INK4B through an Sp1 consensus site. *J. Biol. Chem.* **270**:26750-26753.
29. Liu, F., A. Hata, J. Baker, J. Goody, J. Carcamo, R. Harland, and J. Massague. 1996. A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature* **381**:620-623.
30. Macias-Silva, M., S. Abdollah, P. Hoodless, R. Pirone, L. Attisano, and J. Wrana. 1996. MADR2 is a substrate of the TGF-beta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* **87**:1215-1224.
31. Martin, J. L., M. Ballesteros, and R. C. Baxter. 1992. Insulin-like growth factor-1 (IGF-1) and transforming growth factor-beta1 release IGF-binding protein-3 from human fibroblasts by different mechanisms. *Endocrinology* **131**:1703-1710.
32. Massague, J. 1990. The transforming growth factor-β family. *Annu. Rev. Cell Biol.* **6**:597-641.
33. Massague, J. 1996. TGFbeta signaling: receptors, transducers, and Mad proteins . *Cell* **85**:947-950.
34. Nagatake, M., Y. Takagi, H. Osada, K. Uchida, T. Mitsudomi, S. Saji, K. Shimokata, and T. Takahashi. 1996. Somatic in vivo alterations of the DPC4 gene at 18q21 in human lung cancers. *Cancer Res.* **56**:2718-2720.
35. Newfeld, S. J., E. H. Chartoff, J. M. Graff, D. A. Melton, and W. M. Gelbart. 1996. Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGF-beta responsive cells. *Develop.* **122**:2099-2108.
36. Oh, Y., H. L. Muller, H. Pham, and R. G. Rosenfeld. 1993. Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. *J. Biol. Chem.* **268**:26045-26048.
37. Padgett, R. W., R. D. St.Jonhson, and W. M. Gelbart. 1987. A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family. *Nature* **325**:81-84.

38. Polyak, K., M-H. Lee, H. Erdjument-Bromage, A. Koff, J. M. Roberts, P. Tempst, and J. Massague. 1994. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**:59-66.
39. Riggins, G. J., S. Thiagalingam, E. Rozenblum, C. L. Weinstein, S. E. Kern, S. R. Hamilton, J. K. V. Willson, S. D. Markowitz, K. W. Kinzler, and B. Vogelstein. 1996. Mad-related genes in the human. *Nature Genetics* **13**:347-349.
40. Roberts, A. B. and M. B. Sporn. 1990. The transforming growth factor-beta, p. 419-472. In M. B. Sporn and A. B. Roberts (ed.), Handbook of Experimental Pharmacology, Peptide Growth Factors and their Receptors. Springer, Heidelberg.
41. Savage, C., P. Das, A. L. Finelli, S. R. Townsend, C. -Y. Sun, S. E. Baird, and R. W. Padgett. 1996. Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. *Proc. Natl. Acad. Sci. USA* **93**:790-794.
42. Schutte, M., R. H. Hruban, L. Hedrick, K. R. Cho, G. M. Nadasdy, C. L. Weinstein, G. S. Bova, W. B. Isaacs, P. Cairns, H. Nawroz, D. Sidransky, R. A. Casero, P. S. Meltzer, S. A. Hahn, and S. E. Kern. 1996. DPC4 gene in various tumor types. *Cancer Res.* **56**:2527-2530.
43. Seekelsky, J. J., S. J. Newfeld, L. A. Raftery, E. H. Chartoff, and W. M. Gelbart. 1995. Genetic characterization and cloning of moters against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. *Genetics* **139**:1347-1357.
44. Sekelsky, J. J., S. J. Newfeld, L. A. Raftery, E. H. Chartoff, and W. M. Gelbart. 1995. Genetic characterization and cloning of Mothers against dpp, a gene required for decapentaplegic function in Drosophila meelanogaster. *Genetics* **139**:1347-1358.
45. Sporn, M. B. and A. B. Roberts. 1985. Autocrine growth factors and cancer
Nature **313**:745-47.
46. Sun, L., G. Wu, J. K. V. Willson, E. Zborowska, J. Yang, I. Rajkarunanayake, J. Wang, L. E. Gentry, X. -F. Wang, and M. G. Brattain. 1994. Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *J. Biol. Chem.* **269**:26449-26455.
47. Suzuki, A., C. Chang, J. M. Yingling, X. -F. Wang, and A. Hemmati-Brivanlou. 1997. Smad5 induces ventral fates in Xenopus embryo. *Devel. Bio.* **184**:402-405.
48. Thiagalingam, S., C. Lengauer, F. S. Leach, M. Schutte, S. A. Hahn, J. Overhauser, J. K. V. Willson, S. Markowitz, S. R. Hamilton, S. E. Kern, K. W. Kinzler, and B. Vogelstein. 1996. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nature Genetics* **13**:343-346.
49. Toyoshima, H. and T. Hunter. 1994. p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. *Cell* **78**:67-74.
50. Wall, N. A. and B. L. M. Hogan. 1993. TGFbeta related genes in development. *Curr. Opin. Genet. Dev.* **4**:517-522.
51. Wang, T., P. D. Danielson, B. -Y. Li, P. C. Shah, S. D. Kim, and P. K. Donahoe. 1996. The p21RAS farnesyltransferase a subunit in TGF-beta and activin signaling. *Science* **271**:1120-1122.
52. Wang, T., P. K. Donahoe, and A. Zervos. 1994. Specific interaction of type I receptors of the TGF-beta family with the immunophilin FKBP-12. *Science* **265**:674-676.
53. Wiersdorff, V., T. Lecuit, S. M. Cohen, and M. Mlodzik. 1996. Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the Drosophila eye. *Develop.* **122**:2153-2162.
54. Wu, R. -Y., Y. Zhang, X. -H. Feng, and R. Derynck. 1997. Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. *Mol. Cell Biol.* **17**:2521-2528.
55. Yingling, J. M., P. Das, C. Savage, M. Zhang, R. W. Padgett, and X. -F. Wang. 1996. Mammalian dwarfins are phosphorylated in response to transforming growth factor beta and are implicated in control of cell growth. *Proc. Natl. Acad. Sci. USA* **93**:8940-8944.
56. Zhang, Y., X. -H. Feng, R. -Y. Wu, and R. Derynck. 1996. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* **383**:168-172.

D. APPENDIX

1. Chen, C., Wang, X.-F., Sun, L. (1997) Expression of transforming growth factor beta type III receptor restores autocrine TGFß1 activity in human breast cancer MCF-7 cells. J. Bio. Chem., 272, 12862-12867.

2. Yingling, J. M., Datto, M. B., Wong, C., Frederick, J. P., Liberati, N. T., Wang, X.-F. (1997) The tumor suppressor, Smad4, is a TGF-ß-inducible DNA binding protein. Submitted.

The Tumor Suppressor, Smad4, is a TGF-β-Inducible DNA Binding Protein

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Abstract

Members of the Smad family of proteins are thought to play important roles in transforming growth factor-β mediated signal transduction. In response to TGF-β, specific Smads become inducibly phosphorylated, form heteromers with Smad4, and undergo nuclear accumulation. In addition, over-expression of specific Smad combinations can mimic the transcriptional effect of TGF-β on both the PAI-1 promoter as well as the reporter construct, p3TP-Lux. Although this data suggests a role for Smads in regulating transcription, the precise nuclear function of these heteromeric Smad complexes remains unknown. Here we show that in Mv1Lu cells Smad3 and Smad4 form a TGF-β induced, phosphorylation dependent, DNA-binding complex that specifically recognizes a bipartite binding site within p3TP-Lux. Furthermore, we demonstrate that Smad4 itself is a DNA binding protein which recognizes this same sequence. Interestingly, mutations which eliminate the Smad DNA binding site do not interfere with either TGF-dependent transcriptional activation or activation by Smad3/Smad4 co-overexpression. In contrast, mutation of adjacent AP1 sites within this context eliminates both TGF-β-dependent transcriptional activation and activation in response to Smad3/Smad4 co-overexpression. Taken together, this data suggests that the Smad3/Smad4 complex has at least two separable nuclear functions: it forms a rapid, yet transient sequence-specific DNA-binding complex and it potentiates AP1-dependent transcriptional activation.

Introduction

Transforming growth factor-β (TGF-β) is a multipotent peptide hormone which regulates a diverse array of biological processes (21). The involvement of TGF-β in the pathogenesis of several diseases has resulted in intense investigation of its molecular mechanism of signal transduction (22). Several years ago the signaling receptors for TGF-β were cloned and found to be transmembrane serine/threonine kinases termed the type I and type II receptors (3, 17). Although the molecular nature and mechanism of activation for these TGF-β receptors at the cell surface has been described (28, 29), the intracellular pathways which transduce the TGF-β signal from the membrane to the nucleus has only recently begun to be elucidated.

Genetic studies in *Drosophila* (24) and *C. elegans* (23) identified a conserved family of proteins as playing a critical role in TGF-β superfamily signaling pathways downstream of the receptors. Mammalian homologs of these proteins, now referred to as Smads (8), were subsequently cloned and characterized (1, 20). Studies in *Xenopus* embryos has revealed a functional division between the mammalian Smad proteins. Smad1 (10, 18, 27) and Smad5 (25) have been shown to induce ventral mesoderm and thus mediates the BMP signal, while Smad2 transduces TGF-β signals and induces dorsal mesoderm (2, 10). The distantly related Smad4 protein which was originally identified as a tumor suppressor protein on chromosome 18q (11), induces both ventral and dorsal mesoderm and thus mimics TGF-β and BMP signals (33). Smad4 has been shown to associate with Smad1 in response to BMP and with Smad2 in response to TGF-β and thus is a common component of these signal transduction pathways (15). The Smads have been found to be inducibly phosphorylated in response to TGF-β and BMP and the ligand-specific nature of the Smads have been confirmed by these studies. Smad2 and Smad3 are

specifically phosphorylated in response to TGF-β (9, 16, 31, 32), while Smad1 is phosphorylated in response to BMP (12, 31). Phosphorylation of the Smads results in their heteromerization with Smad4 (15), and correlates with their accumulation in the nucleus (2, 12, 18). Recently, the type I receptor was found to be the kinase responsible for ligand inducible phosphorylation of C-terminal serine residues of Smad1 in response to BMP (14) and Smad2 in response to TGF-β (19). The C-terminal domain of Smad1 and Smad4 has been shown to possess transcriptional activation activity in the context of a Gal4-DNA-binding domain fusion (18), thus providing the first indication of a nuclear function for the Smad proteins. Subsequently, over-expression of specific Smad combinations has been found to mimic the transcriptional effect of TGF-β on both the PAI-1 promoter as well as the reporter construct, p3TP-Lux (5, 15, 18, 19, 32). Smad4 has been shown to be required for this transcriptional activity since Smad4-deficient cell lines are non-responsive, but can be rescued with Smad4 expression (7, 15). Interestingly, the homomeric and heteromeric interactions between Smad3 and Smad4 correlate with their ability to transcriptionally activate the PAI-1 reporter (30). Furthermore, naturally occurring Smad4 mutations interfere with its ability to associate with Smad3 (30). Although these data suggest a role for Smads in regulating transcription, the precise nuclear function of the heteromeric Smad complexes remains unknown.

Here we demonstrate that Smad3 and Smad4 participate in a DNA-binding complex on a fragment of the p3TP-Lux reporter and that Smad4 is the DNA-binding component of this complex. In the context of this reporter, the Smad binding site is not required for transcriptional activation in response to TGF-β nor Smad3/Smad4 co-overexpression. However, we also show that an endogenous promoter, the plasminogen activator inhibitor-1 (PAI-1) promoter, contains a

Smad binding site. Thus, the ability of Smad3/Smad4 to directly bind DNA may have physiological relevance in regulating transcription of TGF-β responsive genes.

Materials and Methods

Cell Culture. Mink lung epithelial cells (Mv1Lu) were obtained from ATCC and maintained in DMEM with 10% FBS, penicillin and streptomycin and non-essential amino acids. COS cells were maintained in DMEM with 10% FBS, penicillin and streptomycin.

Plasmid Construction. Flag-tagged human Smad4, human Smad3 and human Smad2 were the generous gift of Dr. Rik Derynck. Expression vectors for Smad3 WT and Smad3MT (3S→A) were generated by PCR using the following primers: Smad3 WT; 5' primer: GGATCCGCGATGTCGTCCATCCTGCCTTTCAC and 3' primer: GGATCCTAAGACACACTGGAACAGC; Smad3MT (3S→A); 5' primer: same as Smad3 WT above and 3' primer: GGATCCTAAGCCACAGCTGCACAGCGGATGCTTGG. The resulting BamHI fragments were cloned in-frame with the HA tag in pCGN (26). p3TP-Lux (28) and pGL2-T+I have been previously described (6). The luciferase reporter constructs 4X WT, 4X SBS Dbl mutant and 4X AP1 Dbl mutant were created using the following oligos: 4X WT; GGATGAGTCAGACACCTCTGGCTGTCCGGAAG and TCCCTTCCGGACAGCCAGAGGTGTCTGACTCA, 4X AP1 Dbl mutant; GGATACAGCAGACACCTCTGGCTGTCCGGAAG and TCCCTTCCGGACAGCCAGAGGTGTCTGCTGTA, 4X SBS Dbl mutant; GGATGAGTCACTGCATTCTGGCTGTCCGGAAG and

TCCCTTCCGGACAGCCAGAATGCAGTGACTCA. 2X directional constructs were created by first phosphorylating the above oligo sets, annealing and ligating in the presence of 0.5x molar ratio of phosphorylated and annealed linker oligos: short linker, GGCTCGAGAGATCT; long linker 5' : TCCAGATCTCTCGAGCC and long linker 3' : GGAAGATCTCTCGAGCC. The resulting ligation was digested with BglII and cloned into the BglII site of pGL2 T+I. Constructs which contained two inserts in a backwards orientation where then cut with XhoI and EcoRV. The two site containing fragments were then cloned into the XhoI and SmaI sites in reporter constructs which contained two inserts in a forwards orientation to produce constructs with four inserts in the same orientation. GST-Smad3 and GST-Smad4 were created by PCR from plasmid templates using the following primers: Smad3; 5'primer:

CGGGATCCCGATGTCGTCCATCCTGCCTTTCAC, 3' primer : same as Smad3 WT above; Smad4; 5'primer: CGGGATCCCGATGGACAATATGTCTATTACG, 3' primer:

GGATCCTCAGTCTAAAGGTTGTGGG. The resulting BamHI fragments were cloned in-frame into pGex3X-HMK (Pharmacia).

Electrophoretic Mobility Shift Assays. Extracts were prepared from approximately $2x10^6$ COS cells transiently transfected with Smad expression constructs using a standard DEAE-Dextran transfection protocol (28) or from approximately $2x10^6$ Mv1Lu control cells or cells treated with 100 pM TGF-β for 30 min after 2 hours of serum starvation. Cells were then lysed and nuclear extracts were prepared as previously described (6). Whole cell extracts prepared in a lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM DTT, 1 mM PMSF, 1mM sodium orthovanidate, and protease inhibitors, gave identical gel shift results. For phosphatase treatment, 2 U of CIAP (Boehringer Mannheim) and 0.06 U of PAP

(Boehringer Mannheim) were added to 100 ul of nuclear extract (prepared without phosphatase inhibitors) and incubated at 37°C for 30 min in the presence or absence of phosphatase inhibitors; 1mM sodium orthovanidate, 10 mM NaF, 10 mM β-glycerophosphate, and 0.2 mM sodium molybdate. α^{32}P-dTTP Klenow labeled oligos used for probes were as shown in Fig. 2A. Alternatively wild type probe was created by digesting p3TP-Lux with SphI and NdeI and α^{32}P-dTTP Klenow labeling. The PAI-1 promoter probe was obtained by restriction digest with NcoI and EagI and α^{32}P-dTTP Klenow labeling. Gel shift condition were as follows: 1.5 uL of nuclear extract (or 3 ul whole cell extract) containing approximately 3 ug of protein, 1 ug of dIdC and 0.5 ng of probe labeled to an activity of 10,000 - 40,000 cpm/0.5 ng were brought to a final volume of 15 ul using a hypotonic lysis buffer as previously described (6). For supershift analysis a rabbit polyclonal Smad4 antibody was created against full length GST-Smad4 by standard protocols. Pre-immune serum is from the same rabbit. Anti-HA antibody was obtained from Boehringer-Mannheim, the Pan Fos antibody was obtained from Santa Cruz (K-25) and anti-Flag antibody (M2) was obtained from Kodak IBI. 2 ul of each antibody was used for supershifts. For gel shifts with eluted GST-Smad3 and Smad4, approximately 100 ng of protein in 1 ul of a buffer containing 100mM Tris pH 8, 120 mM NaCl, 25 uM glutathione was used. Complexes were resolved on a 6% acrylamide, 0.04% bis-acrylamide, 0.5X TBE gel as previously described (6), except for the HA supershift panel of Figure 2B which was resolved on a 6% acrylamide, 0.2% bis-acrylamide, 0.5X TBE gel.

Methylation Interference. Methylation interference probes were prepared as above with the following exceptions: 4 ul of DMS was added to 100 ul of the Klenow labeling reaction which contained 1 ug total of DNA. After a 5 min room temperature incubation 40 ul of 1.5M sodium

acetate and 1M β-mercaptoethanol was added. Probe was then precipitated with the addition of 0.5 ml of 100% ETOH. Probe was then gel purified and used in EMSA as described above. After a short -80°C exposure of the unfixed/undried EMSA gel, shifted complexes were cut out and bound probe was electro-eluted, precipitated and resuspended in 100 ul of 1M piperidine. Samples were then heated to 90°C for 30 min, and piperidine was subsequently removed by several rounds of lyophilization and resuspension in distilled water. The resulting cleaved products were resolved on a urea/acrylamide sequencing gel.

Luciferase Assays. Transfections were performed using a standard DEAE-Dextran transfection protocol (28). Luciferase assays were performed as previously described (6). All transfections are normalized to β-galactosidase activity by co-transfection of 0.5 ug of a CMV-β-gal expression vector . Quantities of DNA transfected are detailed in the figure legends.

Western Blot Analysis. Proteins from COS transfected lysates were resolved by 8% SDS-PAGE and transferred to Immobilon (Millipore). The blots were blocked in B/P solution (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 2% milk. Primary antibody (HA, Flag or Smad4) was added in B/P solution at 1:1000 for 1 hr at room temperature. The blots were washed 3X with B/P solution and the appropriate secondary antibody (Bio-Rad) was added (goat anti-mouse for HA/Flag and goat anti-rabbit for Smad4) for 1 hr at room temperature. After washing 3X with B/P solution, the blots were developed with ECL (Amersham) and exposed on Kodak XAR5 film.

Results

Smad3/Smad4 Co-Overexpression Regulates Transcription. The p3TP-Lux luciferase reporter is a well described and widely used artificial promoter construct which was empirically designed to have maximal responsiveness to TGF-β (28). p3TP-Lux has a 31-nucleotide, AP1 site containing region of the collagenase promoter, concatamerized 5' to an ~400 nucleotide region of the PAI-1 promoter followed by 70 bp of the adenovirus E4 promoter (Fig. 1A). Consistent with previous findings, we observe a transcriptional activation of p3TP-Lux by both Smad3/Smad4 co-overexpression and TGF–β treatment in Mv1Lu cells (Fig. 1B). In contrast, Smad2 fails to activate transcription of p3TP-Lux when co-overexpressed with Smad4. To define the Smad responsive region of p3TP-Lux, we created a reporter construct comprised only of the 31-nucleotide AP1 site containing region concatamerized 5' to a minimal promoter. This 4X WT reporter (Fig. 1A) is not only TGF-β-responsive, but is also activated in response to Smad3/Smad4 co-overexpression (Fig. 1C). Thus, this 31-nucleotide repeat contains a DNA sequence which is both TGF-β and Smad responsive.

Smad3/Smad4 Participates in a DNA-Binding Complex. To determine if Smad3/Smad4 co-overexpression changes the DNA-binding complexes on this 31-nucleotide fragment, we performed gel shifts using a probe consisting of two copies of the 31-nucleotide repeat cut from p3TP-Lux, termed the 2.0 probe (Fig. 3A). When gel shifts were performed using this probe and extracts derived from COS cells co-transfected with epitope tagged Smad3 and Smad4 (Fig. 2A), we observed not only an AP1 containing complex (Complex I), but also a strong additional binding complex (Complex II, Lane 6). Overexpression of Smad3 alone produces a lower level of a complex with similar mobility (Lane 3). Likewise, overexpression of Smad4 produces a

complex with similar mobility, as well as a slightly faster migrating complex (Lane 4). In contrast, Smad2/Smad4 co-expression does not produce this complex, but appears similar to Smad4 alone (Lane 5).

One possible explanation for these observations is that Smad3 and Smad4 form a DNA binding complex. Overexpressed Smad3 alone or Smad4 alone could bind DNA with their endogenous Smad partner, whereas co-overexpression would produce a large amount of Smad3/Smad4 binding complex. To test this hypothesis, supershift analysis was performed to determine if HA-tagged Smad3 or Flag-tagged Smad4 are present in the additional binding complex (Complex II). As shown in Figure 2B, both HA and Flag antibodies supershift this complex (Lanes 7, 12 and 13). As expected, a Pan-Fos family member antibody supershifts the faster migrating AP1 complex (Lane 2). This antibody, however, does not shift the Smad3/Smad4 complex (Lane 8), suggesting that although the constitutive binding activity contains a Fos family member, the Smad3/Smad4 complex does not. Finally, the complexes observed with Smad4 overexpression are all Smad4 containing as demonstrated by Flag supershifts (Lane 5). Thus, Smad3 and Smad4, when overexpressed, participate in a DNA binding complex on sequences present in this region of p3TP-Lux.

Recently, the BMP-inducible phosphorylation sites of Smad1 and the TGF-β-inducible phosphorylation sites of Smad2 have been identified (14, 19). Smad3 contains analogous sites of potential phosphorylation at its C-terminus. Based on this sequence homology, we created a phosphorylation deficient mutant of Smad3, Smad3MT, and assayed its ability to participate with Smad4 in a DNA binding complex. Although the expression levels were similar to wild-type Smad3, Smad3MT was unable to form a DNA binding complex with Smad4 (Fig. 2A, Lane 7). The results with this mutant suggest that an intact carboxyl-terminus of Smad3 is essential for

formation of the DNA binding complex. This mutation possibly interferes with the ability of Smad3 to form a heteromeric complex with Smad4 and thus precludes formation of the DNA-binding complex.

Isolation of the Smad DNA-Binding Element. To more precisely determine the DNA sequences to which the Smad3/Smad4-containing complex binds, we systematically mutated the 2.0 probe (Fig. 3A). As expected, mutation of the AP1 binding sites eliminated the Fos-containing shifted complex. The Smad3/Smad4 complex, however, was still present on the AP1 site mutant probe, although in somewhat decreased amounts (Fig. 3B, Lane 3). This further suggests that the Smad3/Smad4 complex is not binding through AP1. We next designed three separate scanning mutants to encompass the entire 2.0 probe in search of the specific sequence which confers Smad3/Smad4 binding (Fig. 3A). As shown in Figure 3B, scanning mutant #1 eliminates both the AP1 and the Smad3/Smad4 complexes, while scanning mutant #2 specifically eliminates the Smad3/Smad4 complex leaving the AP1 complex intact. Scanning mutant #3 has no effect on the binding of either complex. Thus, the region necessary for Smad3/Smad4 complex binding lies within the bases mutated in scanning mutants #1 and #2.

Methylation interference was used to more precisely define which guanine residues within the 2.0 probe are contacted by the Smad3/Smad4 complex. The results shown in Figure 3C confirm the mutagenesis results in that there is a single protected guanine residue that is located within the region predicted by the scanning mutagenesis. Both sites of this two site probe have almost completely protected guanine residues. This suggests that both sites are being contacted in this single Smad3/Smad4 complex. Mutation of 6 nucleotides surrounding this protected guanine (GACACC) in either the 5' or 3' site of the 2.0 probe was sufficient to

eliminate Smad3/Smad4 binding (Fig. 3D), further indicating the requirement of a bipartite site for Smad3/Smad4 complex formation. In addition, a probe containing only one of these 31-nucleotide repeats (one half of the probe used in these experiments) was completely unable to bind the Smad3/Smad4 complex in gel shift assays (data not shown).

Smad4 Directly Binds DNA. Having demonstrated that overexpressed Smad3/Smad4 participates in a DNA-binding complex on the 2.0 probe, we next sought to determine if either Smad3 or Smad4 themselves were directly binding this DNA sequence. Therefore, we generated GST fusions of both proteins and used these purified reagents in gel shifts with the 2.0 probe (Fig. 4). Although Smad3 is incapable of binding (Lanes 1 and 2), GST-Smad4 directly binds the 2.0 wild type (Lanes 3 and 4) and AP1 mutant probes (Lane 6), but does not bind the 2.0 Smad binding site mutant probe (Lane 5). The DNA-binding protein was confirmed to be Smad4 by antibody supershift analysis. The Smad4 specific immune antisera alone produces a background DNA-binding band (Lane 7). The Smad4 antibody eliminates the 2 specific DNA-binding complexes (Lane 9), while the preimmune serum has no effect (Lane 10). Thus, the complex seen on Smad3/Smad4 co-overexpression may be the result of a direct DNA interaction by Smad4. Although Smad4 binds directly to this DNA sequence, Smad3 may modulate its binding affinity or affect its binding specificity. An altered specificity of Smad4 in complex with different Smads may explain why Smad2/Smad4 complexes do not bind this sequence, but Smad3/Smad4 complexes do. This altered specificity of Smad4 DNA-binding in complex with different Smads would be required to maintain the specific transcriptional events that occur in response to TGF-β superfamily ligands. The ability of Smad4 to directly bind DNA explains the additional shifted complex observed when Smad4 is overexpressed alone in COS cells (Fig. 2A

and B, Lanes 4); it is Smad4 bound without endogenous Smad3. The ability of the Flag antibody to supershift this complex confirms the presence of Smad4 in this complex (Fig. 2B, Lane 5).

TGF-β Induces a Smad DNA-Binding Complex *In Vivo*. Mv1Lu cells are highly responsive to TGF-β and have been used as a model system to define various aspects of TGF-β mediated signal transduction. Thus, we used Mv1Lu cells as a model system to look *in vivo* for a Smad containing DNA-binding complex. Since the Smad proteins are known to be cytoplasmic proteins which translocate to the nucleus in response to ligand-induced phosphorylation, an endogenous Smad-containing DNA-binding complex would be predicted to be TGF-β-inducible and phosphorylation dependent. To examine this question, we performed gel shifts with the 2.0 probe and nuclear extracts prepared from either TGF-β treated or untreated Mv1Lu cells. In the absence of TGF-β treatment, Mv1Lu cells contain a constitutive Fos-containing binding complex similar to the Fos complex in COS cells (Fig. 5C, Lane 2). Upon TGF-β treatment, a slower migrating complex appears within 5 min, peaks in 15 min and disappears after 4 hrs. (Fig. 5A). This time course parallels the TGF-β-dependent phosphorylation kinetics of endogenous Smad proteins (31). In addition, the inducibly bound complex is sensitive to phosphatase treatment, suggesting that its binding is phosphorylation dependent (Fig. 5B). Thus this inducible complex has the characteristics expected for a Smad-containing DNA-binding complex. The presence of Smad4 in this TGF-β inducible complex was confirmed by the ability of a Smad4-specific antibody to eliminate formation of this complex (Fig. 5C, Lane 5).

Unfortunately, our Pan-Smad antibodies which recognize Smad1, 2, 3 and 5 (31) could not supershift either the endogenous Smad4-containing complex nor the Smad3/Smad4 co-overexpressed complex from COS cells because of their relatively low affinity for Smad3 (data

not shown). Therefore, we cannot unequivocally show that Smad3 is a component of the TGF-β-inducible shifted complex in Mv1Lu cells. However, the inducible complex comigrates with the Smad3/Smad4 complex from COS lysates (data not shown) and shares an identical binding site within the 2.0 probe as revealed by gel shift analysis using the panel of 2.0 probe mutants (Fig. 5D). These data combined with the fact that no other Smad in combination with Smad4 from COS lysates is able to bind the 2.0 probe, provides strong evidence that the inducible complex in Mv1Lu cells contains Smad3 and Smad4.

Functional Analysis of the Smad-DNA-Binding Element. Having identified the specific region of the 2.0 probe which was capable of conferring Smad3/Smad4 binding, we examined the functional consequences of Smad binding site and AP-1 site mutations in the context of the 4X WT reporter in Mv1Lu cells. As shown in Figure 6, the AP1 sites are critically important for induction by both TGF-β and Smad3/Smad4 co-overexpression. Surprisingly, mutation of the Smad binding site had no effect on induction by TGF-β or by Smad3/Smad4 co-overexpression. These results suggest that the heteromeric Smad3/Smad4 complex has at least two distinct nuclear activities. First, it rapidly forms a transient, sequence-specific DNA-binding complex with unknown function and secondly, it directly or indirectly potentiates AP1-dependent transcriptional regulation in the context of the p3TP-Lux reporter.

The Endogenous PAI-1 Promoter Contains a Smad DNA-Binding Element. The p3TP-Lux reporter is an artificial construct designed empirically for maximum TGF-β responsiveness. Although it has been instructive to biochemically define a novel binding function for Smad complexes, the question remains; does this Smad complex form on endogenous or native

promoter sequences? To address this question, we examined endogenous promoters that are known to be activated by TGF-β and Smad co-overexpression for their ability to bind a Smad3/Smad4 containing complex. One such DNA sequence is an 800 bp stretch of the PAI-1 promoter. The TGF-β responsive region of the PAI-1 promoter has been described and surrounds a putative AP1 binding site (Fig. 7A and 13). The similarity of this endogenous sequence to that created in p3TP-Lux made it an ideal candidate for study. Using a probe that encompasses this AP1 site, we discovered that indeed Smad3 and Smad4 co-overexpression in COS cells leads to an additional DNA-binding complex (Fig. 7B, Complex II). Thus, the ability of Smad3/Smad4 to bind DNA which we originally defined in the context of p3TP-Lux may have physiological relevance in TGF-β's ability to regulate endogenous promoters such as PAI-1. Studies are currently underway to investigate the functional role of this Smad-binding region within the PAI-1 promoter.

Discussion

An intracellular pathway for mediating TGF-β superfamily signals from the membrane to the nucleus has begun to be elucidated. This highly conserved pathway involves the Smad proteins, which have been shown to be phosphorylated by the type I receptor within the heteromeric signaling complex at the cell surface, to form heteromers with Smad4 and to accumulate in the nucleus. In this study, we have investigated the molecular nature of the Smads ability to transcriptionally activate the p3TP-Lux reporter.

Overexpression of Smad3 and Smad4 was found to activate transcription from p3TP-Lux in a ligand-independent fashion in Mv1Lu cells. Overexpression of Smad3/Smad4 results in the formation of a specific DNA-binding complex on the responsive region of the promoter. The

inability of Smad2 and Smad4 to form a similar DNA-binding complex on this region correlates with their failure to activate transcription from the p3TP-Lux promoter. Thus, the heteromeric Smad4 complexes which are formed as a result of ligand-induced phosphorylation of Smad2 and Smad3 may have distinct nuclear targets. Recently, Smad2 has been found in a DNA binding complex with the transcription factor, FAST1 (4). Although the presence of Smad4 in this complex has not yet been reported, these findings, in combination with our data, raises the possibility that different Smad complexes will target different sequences to differentially affect distinct subsets of genes. In the FAST1 context, FAST1 may be making the sequence specific DNA interaction, tethering the Smad complex to the promoter, whereas in p3TP-Lux and PAI-1, Smad4 appears to be the specific DNA-binding protein. This level of complexity may provide the diversity necessary for the regulation of a broad set of TGF-β responsive genes.

Detailed mutagenesis and methylation interference analysis of the responsive region identified a bipartite sequence as the Smad-binding site. The only apparent similarity of this binding site to that previously identified for the FAST-1/Smad2 complex is its bipartite nature. Mutation of a small, 6-nt region, was sufficient to eliminate Smad3/Smad4 binding to this probe. Functional assays with mutant reporters which lack AP1 or Smad-binding sites, revealed that the AP1 sites are required for TGF-β and Smad3/Smad4-dependent transcriptional activation. In contrast, the Smad-binding site we defined in p3TP-Lux was dispensable for both TGF-β and Smad3/Smad4-dependent transcriptional activation. The apparent dispensability of the Smad binding site within this reporter could be explained in several ways. Smad complex binding may be having effects which are not assayed in these transient transfection experiments. If, for example, Smad binding plays a role in the recruitment of other transcription factors to adjacent sites (e.g. AP1), or in re-arrangement of chromosome structure to provide accessibility of other

transcription factors to their binding sites, an effect in the transient transfection assay may be difficult to observe. The transient nature of Smad DNA-binding would be consistent with this type of role in transcriptional activation. Alternatively, in the context of our artificial promoter constructs, Smad binding may not be required, but in other promoter contexts, Smad binding may be essential. Our demonstration that an endogenous promoter, PAI-1, contains a Smad3/Smad4 binding site provides an opportunity to dissect *in vivo* functions of the Smad3/Smad4 binding site and should provide insight into these important questions.

Although the functional consequences of Smad binding remains uncertain, we have clearly demonstrated that Smad3/Smad4 co-overexpression can activate transcription through AP1 binding sites. This raises the possibility that the Smads have at least two separable functions. One is a direct effect through its sequence specific DNA binding. The second is a potentially more indirect effect to activate AP1-mediated transcription, and may explain the widely observed phenomenon that TGF-β can activate transcription through AP1 binding sites.

Finally, we present evidence that Smad4 is a DNA binding protein. Obviously, more work is required to determine if this function of Smad4 is at the root of its tumor suppressor activity. For example, defining the domain of Smad4 required for its DNA-binding ability and determining if known Smad4 mutations interfere with this activity. However, our results do raise the exciting possibility that Smad4 functions by targeting distinct heteromeric Smad complexes to various promoters to affect their transcription. Identifying these Smad4 binding site targets could greatly enhance our understanding of the function of this protein, and of its role as a tumor suppressor.

Figure Legends

Figure 1. Smad overexpression regulates transcription. (A) Diagram of the AP1 containing luciferase reporters. (B) p3TP-Lux is activated both by TGF-β and by Smad3/Smad4 co-transfection. Briefly, $2x10^6$ Mv1Lu cells were co-transfected with 3 ug of p3TP-Lux, 0.5 ug pCMV β-gal and either 2 ug pCGN vector (M) or 1 ug of the indicated Smad and 1 ug pCGN (2, 3, 4) or 1 ug Smad4 and 1ug of either Smad2 or Smad3 (2/4, 3/4). 12 hours after transfection 100 pM TGF-β was added and TGF-β induced luciferase activity was assayed 20-24 hours later. (C) The 4X WT reporter is activated by TGF-β and Smad3/Smad4 co-expression like p3TP-Lux. Luciferase assays were performed as in (B) with co-transfection of 3 ug of the minimal TATA-INR reporter construct or the 4X WT reporter construct with vector alone (M) or the combination of Smad3 and Smad4 (3/4). Luciferase assays were performed in duplicate at least three times and are standardized against β-gal expression as an internal control.

Figure 2. Co-overexpression of Smad3 and Smad 4 in COS cells changes the 2.0 probe binding profile. (A) Gel shifts were performed using the 2.0 probe derived from p3TP-Lux and extracts derived from COS cells transiently transfected with either 7 ug of pCGN (M, Lane 1), 2 ug of Smad2 (2, Lane 2) or Smad3 (3, Lane 3), 5 ug of Smad4 (4, Lane 4), or 2 ug of Smad2, 3, or 3MT in combination with 5 ug of Smad4 (2/4, Lane 5; 3/4, Lane 6; 3MT/4, Lane 7). DNA amount was kept constant at 7 ug with added pCGN vector. An HA/Flag western blot was performed to confirm expression of these proteins (Inset panel). (B) Smad 3 and Smad4 participate in a binding complex. Supershifts using antibodies described in the methods were

performed on COS cells transiently transfected as in (A) with pCGN vector alone (Mock), Smad4-Flag (Flag 4), HA-Smad3/Smad4-Flag (HA 3/Flag 4). Fos: Pan Fos antibody; Fg: Flag epitope antibody; HA: anti-HA epitope antibody.

Figure 3. Identification of the Smad DNA-binding element in the 2.0 probe. (A) Diagram of the 2.0 wild type probe and mutants. Lower case letters indicate mutations introduced into the wild-type nucleotide sequence. (B) Identification of the Smad DNA-binding region. Gel shifts were performed using the indicated probes as diagrammed in (A) and COS cells transiently transfected as in Figure 2 with pCGN vector alone (M) or HA-Smad3/Smad4-Flag (3/4). (C) Methylation implicates a guanine residue within scanning mutant #2 as binding the Smad3/Smad4 complex. Methylation interference was performed using methylated 2.0 probe and COS cells transiently co-transfected with HA-Smad3 and Smad4-Flag as in Figure 2. The protected guanine residues are indicated by a • in the diagram in Figure 3A. (D) Both sites on the 2.0 repeat sequence are required for Smad3/Smad4 binding. Gel shifts were performed using the indicated probes as diagrammed in (A) and COS cells transiently transfected with pCGN vector alone (M) or HA-Smad3/Smad4-Flag (3/4).

Figure 4. Purified GST-Smad4, but not GST-Smad3 directly binds DNA. GST-fusion protein construction is described in *Materials and Methods*. Gel shifts were performed with 2 ul (Lanes 1 and 3) or 1 ul (Lanes 2 and 4-10) of either GST-Smad3 (Lanes 1 and 2) or GST-Smad4 (Lanes 3-10) at a concentration of ~100 ng/ul and the indicated probes as diagrammed in Fig. 3A and the following antibodies: S4, Smad4 antibody; Pre, preimmune serum.

Figure 5. Induction of a Smad3/Smad4 DNA-binding complex in Mv1Lu cells by TGF-β. (A) Gel shift analysis with the 2.0 probe with lysates from a time course of TGF-β treatment of Mv1Lu cells. (B) The inducible complex is sensitive to phosphatase treatment. Mv1Lu lysates from control (Lanes 1, 3 and 5) or TGF-β treated (Lanes 2, 4 and 6) cells were treated with PAP+CIAP (Lanes 1-4) or phosphatase inhibitors (Lanes 3-6) as described in *Materials and Methods*. (C) Supershift analysis on the induced complex in Mv1Lu cells. Gel shifts were performed with the 2.0 probe as diagrammed in Fig. 3A and the indicated antibodies: Fos, Pan Fos antibody; S4, Smad4 antibody; Pre, preimmune serum. Bottom panel, Western showing the specificity of the Smad4 antibody. Left panel is an HA antibody Western showing expression of Smad1-4; right panel is a duplicate Western blot with Smad4 antibody. The Smad4 antibody is also specific by immunoprecipitation analysis (data not shown). (D) Analysis of the 2.0 probe mutants with TGF-β treated Mv1Lu cell lysates. Lysates were prepared from control (odd numbered lanes) or TGF-β treated cells (even numbered lanes) and probes indicated are as shown in Fig. 3A.

Figure 6. Functional analysis of the 2.0 Probe mutants. Mv1Lu cells were transfected with 2 ug vector DNA (M) or with 1 ug HA-Smad3 and Smad4-Flag DNA (3/4) along with 3 ug of the indicated reporter and 0.5 ug pCMV β-gal. Luciferase assays were performed as described in Figure 1.

Figure 7. Endogenous PAI-1 Promoter Contains a Smad3/Smad4 Binding Site. (A) Schematic diagram of the PAI-1 promoter. The black boxes represent potential AP1 binding sites. The shaded region (-792 to -644) represents the probe used in gel shift assays. (B) Gel shift analysis using the probe from (A) was performed with COS lysates from cells transfected as in Fig. 2 with pCGN vector alone (left lane) or HA-Smad3/Smad4-Flag (right lane).

Acknowledgments

This work was supported by grant DK-45746 from the National Institutes of Health to X.-F.W.; U.S. Army Breast Cancer Research grant DAMD17-945-4065 to X.-F.W.; a predoctoral U.S. Army Breast Cancer Research grant DAMD17-94-J-4190 to J.M.Y.; and a predoctoral NSF grant to N.T.L. X.-F.W. is a Leukemia Society Scholar.

The first three authors contributed equally to this work. We wish to thank Rik Derynck for his generous gift of hSmad2, hSmad3 and hSmad4. TGF-β1 was kindly provided by Amgen, Inc. We appreciate helpful discussions with members of the Wang and Pendergast labs and thank Yong Yu for technical assistance.

References

1. **Attisano, L., and J. L. Wrana.** 1996. Signal transduction by members of the transforming growth factor-β superfamily. Cytokine & Growth Factor Reviews **7:**327-339.

2. **Baker, J., and R. Harland.** 1996. A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. Genes & Development **10:**1880-1889.

3. **Bassing, C. H., J. M. Yingling, D. J. Howe, T. Wang, W. W. He, M. L. Gustafson, P. Shah, P. K. Donahoe, and X. F. Wang.** 1994. A transforming growth factor beta type I receptor that signals to activate gene expression. Science **263:**87-9.

4. **Chen, X., M. J. Rubock, and M. Whitman.** 1996. A transcriptional partner for MAD proteins in TGF-beta signalling. Nature **383:**691-6.

5. **Chen, Y., J. J. Lebrun, and W. Vale.** 1996. Regulation of transforming growth factor beta- and activin-induced transcription by mammalian Mad proteins. Proc Natl Acad Sci U S A **93:**12992-7.

6. **Datto, M. B., Y. Yu, and X. F. Wang.** 1995. Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. J Biol Chem **270:**28623-8.

7. **de Caestecker, M. P., P. Hemmati, S. Larisch-Bloch, R. Ajmera, A. B. Roberts, and R. J. Lechleider.** 1997. Characterization of Functional Domains within Smad4/DPC4. J. Biol. Chem. **271:**13690-13696.

8. **Derynck, R., W. M. Gelbart, R. M. Harland, C. H. Heldin, S. E. Kern, J. Massague, D. A. Melton, M. Mlodzik, R. W. Padgett, A. B. Roberts, J. Smith, G. H. Thomsen,**

B. Vogelstein, and X. F. Wang. 1996. Nomenclature: vertebrate mediators of TGF-beta family signals. Cell **87:**173.

9. **Eppert, K., S. W. Scherer, H. Ozcelik, R. Pirone, P. Hoodless, H. Kim, L. C. Tsui, B. Bapat, S. Gallinger, I. L. Andrulis, G. H. Thomsen, J. L. Wrana, and L. Attisano.** 1996. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell **86:**543-52.

10. **Graff, J. M., A. Bansal, and D. A. Melton.** 1996. Xenopus Mad proteins transduce distinct subsets of signals for the TGF beta superfamily. Cell **85:**479-87.

11. **Hahn, S. A., M. Schutte, A. T. Hoque, C. A. Moskaluk, L. T. da Costa, E. Rozenblum, C. L. Weinstein, A. Fischer, C. J. Yeo, R. H. Hruban, and S. E. Kern.** 1996. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science **271:**350-3.

12. **Hoodless, P. A., T. Haerry, S. Abdollah, M. Stapleton, O. C. MB, L. Attisano, and J. L. Wrana.** 1996. MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell **85:**489-500.

13. **Keeton, M. R., S. A. Curriden, A. J. van Zonneveld, and D. J. Loskutoff.** 1991. Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor beta. J Biol Chem **266:**23048-52.

14. **Kretzschmar, M., F. Liu, A. Hata, J. Doody, and J. Massague.** 1997. The TGF-ß family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. Genes & Development **11:**984-995.

15. **Lagna, G., A. Hata, A. Hemmati-Brivanlou, and J. Massague.** 1996. Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. Nature **383:**832-6.

16. **Lechleider, R. J., M. P. de Caestecker, A. Dehejia, M. H. Polymeropoulos, and A. B. Roberts.** 1996. Serine phosphorylation, chromosomal localization, and transforming growth factor-beta signal transduction by human bsp-1. J Biol Chem **271:**17617-20.

17. **Lin, H. Y., X. F. Wang, E. Ng-Eaton, R. A. Weinberg, and H. F. Lodish.** 1992. Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase [published erratum appears in Cell 1992 Sep 18;70(6):following 1068]. Cell **68:**775-85.

18. **Liu, F., A. Hata, J. C. Baker, J. Doody, J. Carcamo, R. M. Harland, and J. Massague.** 1996. A human Mad protein acting as a BMP-regulated transcriptional activator. Nature **381:**620-3.

19. **Macias-Silva, M., S. Abdollah, P. Hoodless, R. Pirone, L. Attisano, and J. Wrana.** 1996. MADR2 is a substrate of the TGF-β Receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell **87:**1215-1224.

20. **Massague, J.** 1996. TGFbeta signaling: receptors, transducers, and Mad proteins. Cell **85:**947-50.

21. **Massague, J.** 1990. The transforming growth factor-β family. Annu.Rev.Cell Biol. **6:**597-641.

22. **Roberts, A. B., and M. B. Sporn.** 1993. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). Growth Factors **8:**1-9.

23. **Savage, C., P. Das, A. L. Finelli, S. R. Townsend, C. Y. Sun, S. E. Baird, and R. W. Padgett.** 1996. Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. Proc Natl Acad Sci U S A **93:**790-4.

24. **Sekelsky, J. J., S. J. Newfeld, L. A. Raftery, E. H. Chartoff, and W. M. Gelbart.** 1995. Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics **139:**1347-58.

25. **Suzuki, A., C. Chang, J. M. Yingling, X.-F. Wang, and A. Hemmati-Brivanlou.** 1997. Smad5 induces ventral fates in *Xenopus* embryo. Developmental Biology **184:**402-405.

26. **Tanaka, M., and W. Herr.** 1990. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell **60:**375-86.

27. **Thomsen, G. H.** 1996. Xenopus mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. Development **122:**2359-66.

28. **Wrana, J. L., L. Attisano, J. Carcamo, A. Zentella, J. Doody, M. Laiho, X. F. Wang, and J. Massague.** 1992. TGF beta signals through a heteromeric protein kinase receptor complex. Cell **71:**1003-14.

29. **Wrana, J. L., L. Attisano, R. Wieser, F. Ventura, and J. Massague.** 1994. Mechanism of activation of the TGF-beta receptor. Nature **370:**341-7.

30. **Wu, R. Y., Y. Zhang, X. H. Feng, and R. Derynck.** 1997. Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. Mol Cell Biol **17:**2521-8.

31. **Yingling, J. M., P. Das, C. Savage, M. Zhang, R. W. Padgett, and X. F. Wang.** 1996. Mammalian dwarfins are phosphorylated in response to TGF-β and are implicated in control of cell growth. Proc Natl Acad Sci U S A **93:**8940-8944.

32. **Zhang, Y., X. Feng, R. We, and R. Derynck.** 1996. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. Nature **383:**168-72.

33. **Zhang, Y., T. Musci, and R. Derynck.** 1997. The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function. Curr Biol **7:**270-6.

Figure 1

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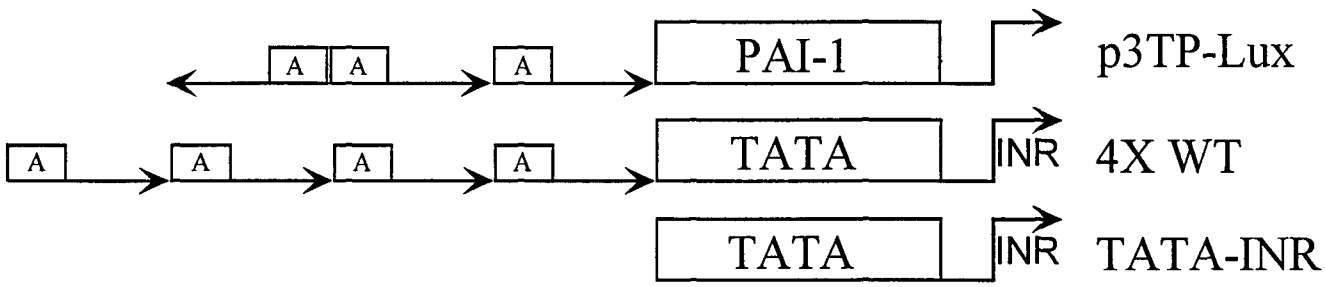

Figure 1

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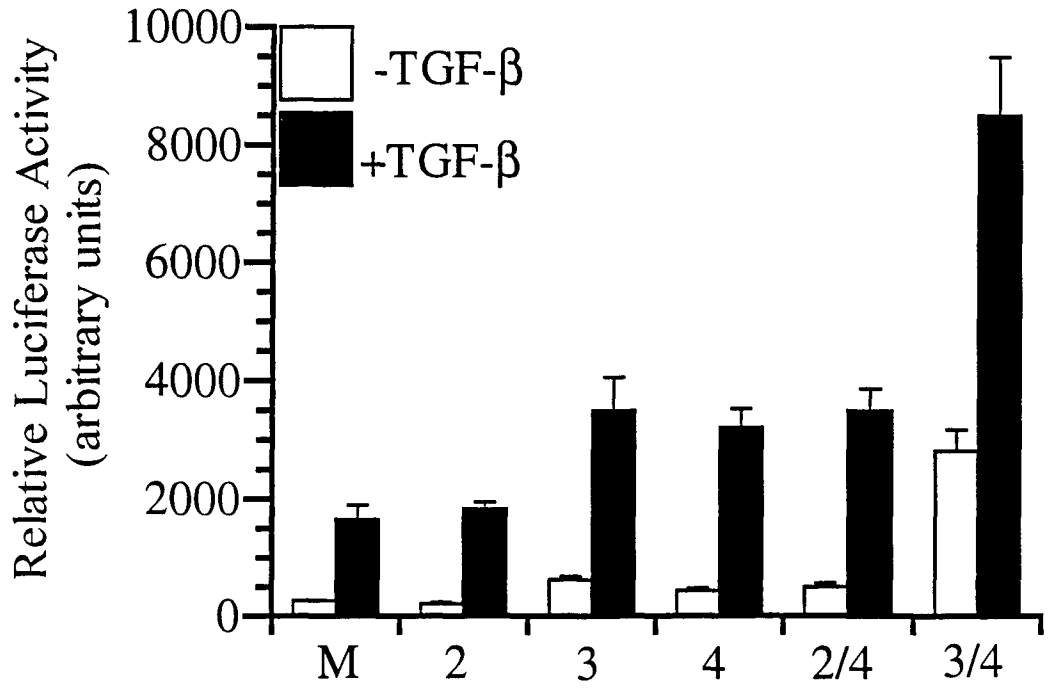
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3/4

C

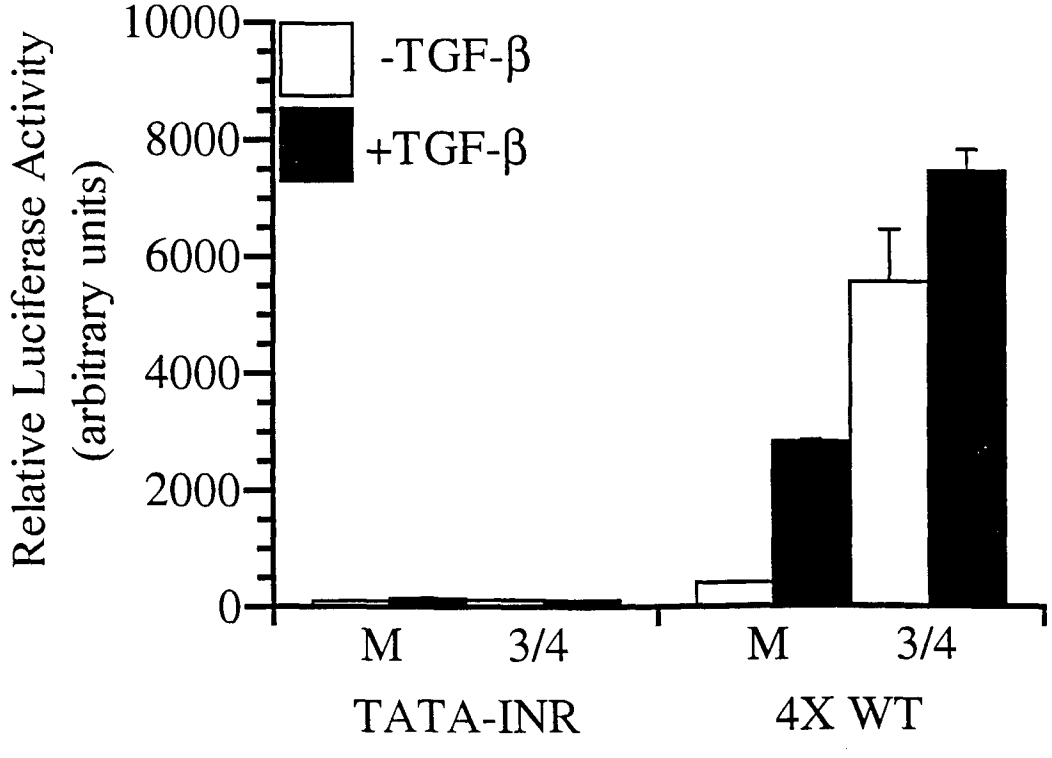
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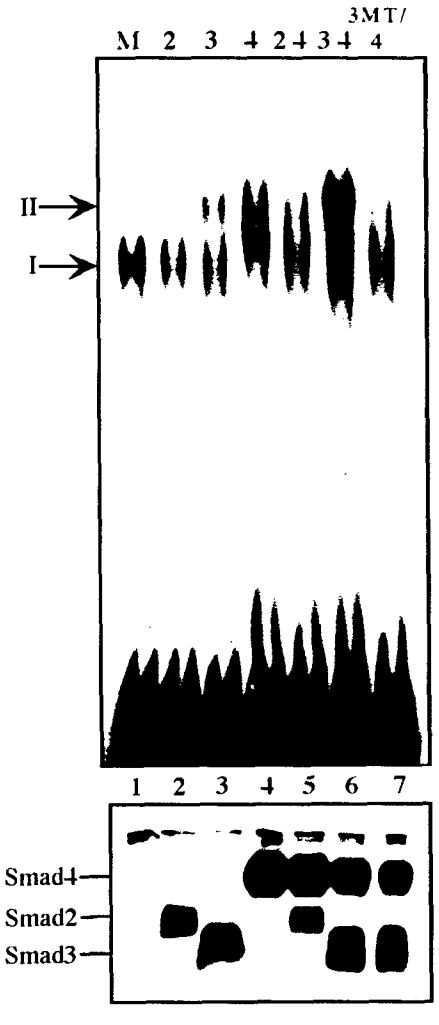
Figure 2

Figure 2A



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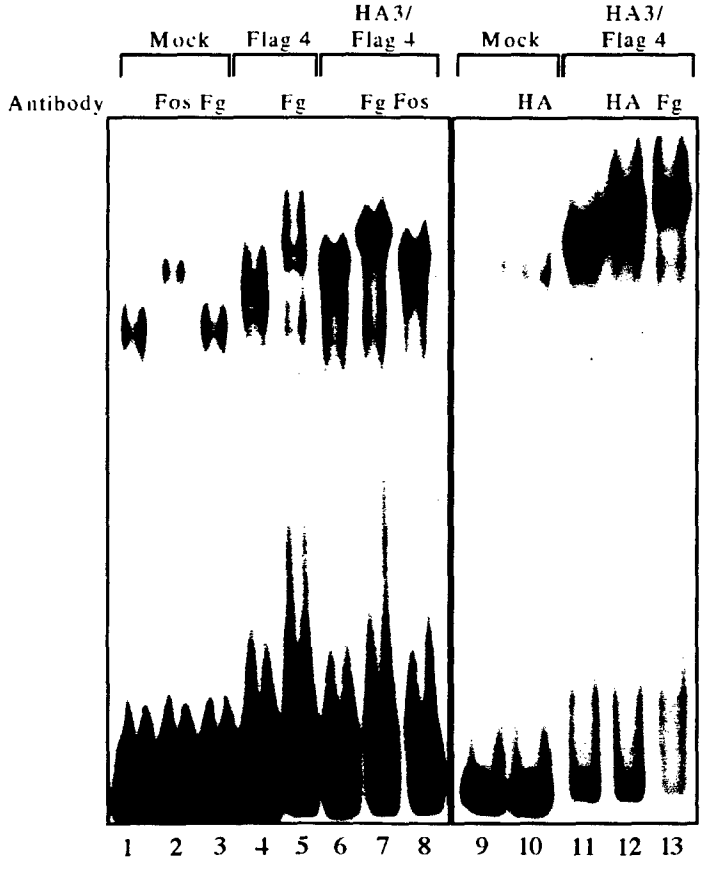

Figure 3

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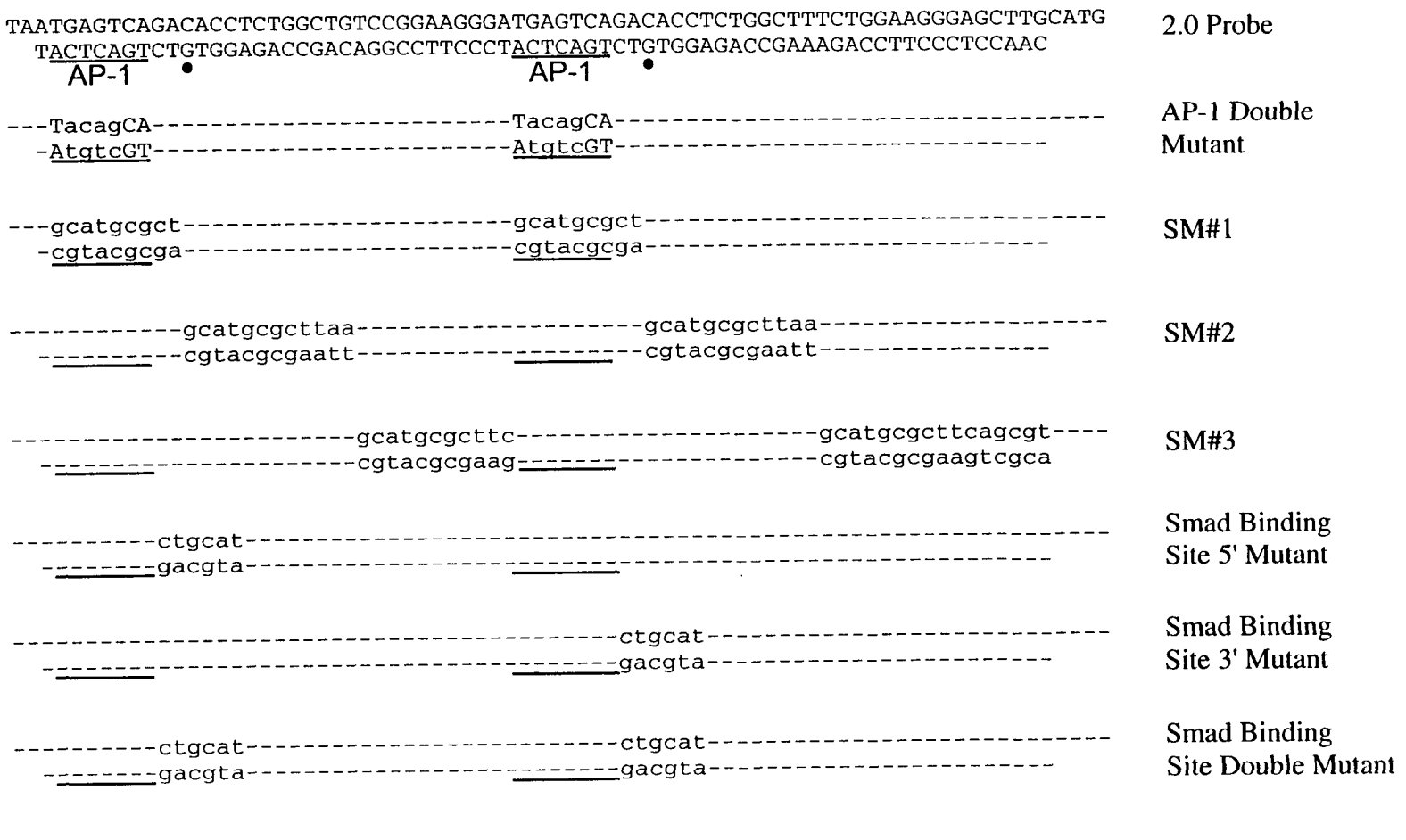
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AP-1
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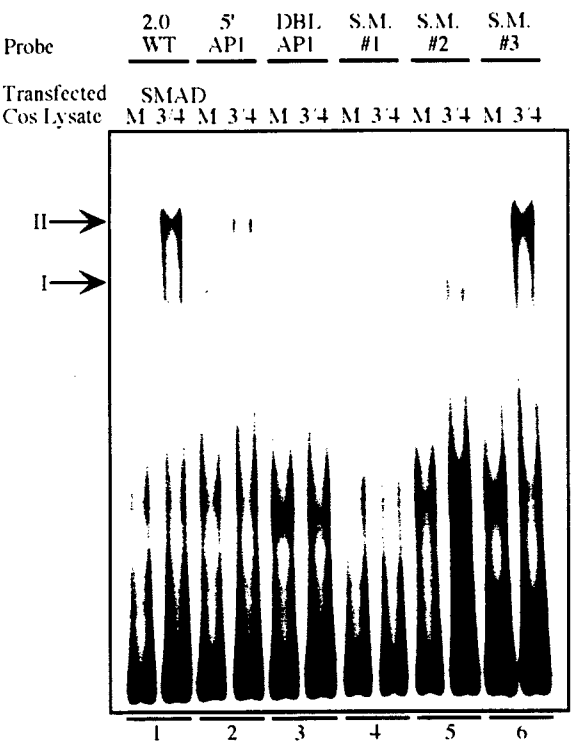
Figure 3B

B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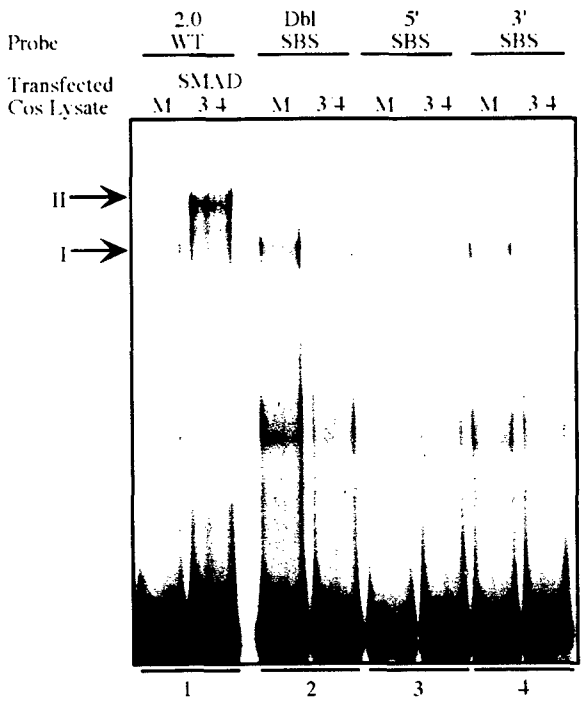
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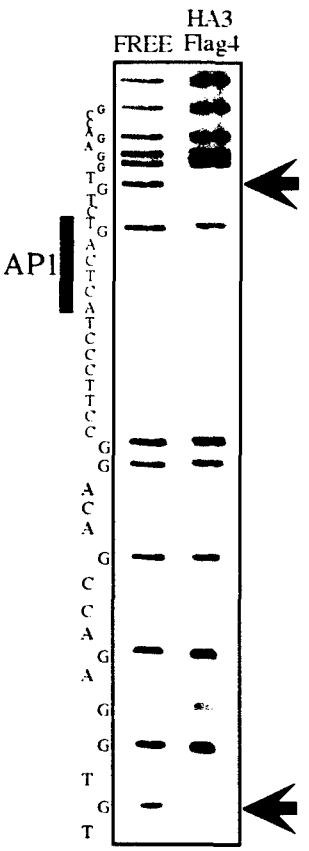
Figure 3C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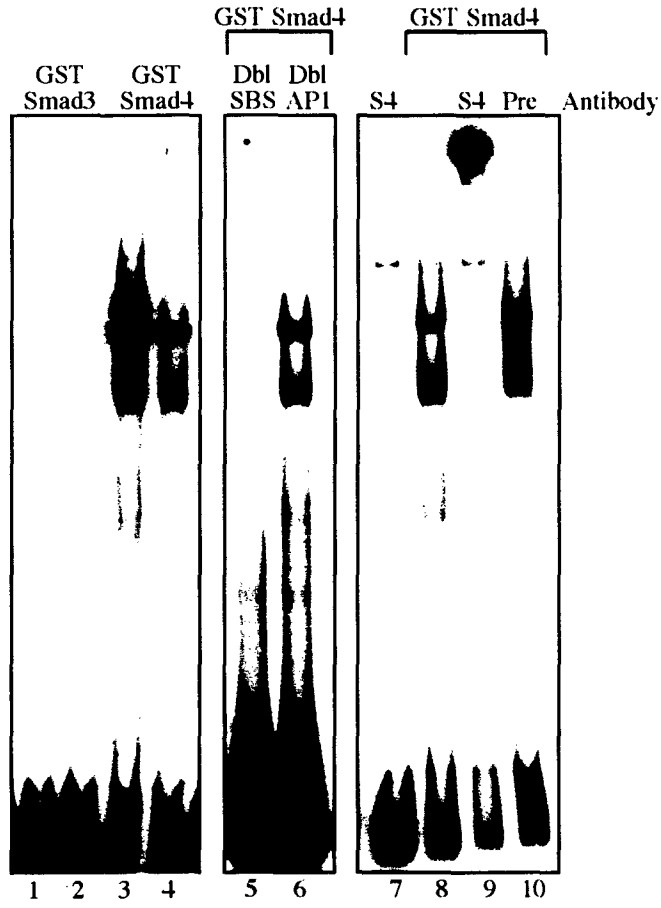
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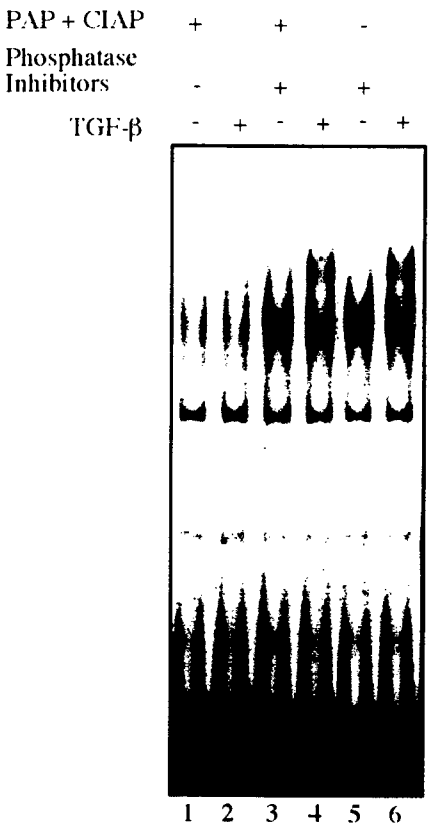
Figure 5

B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A

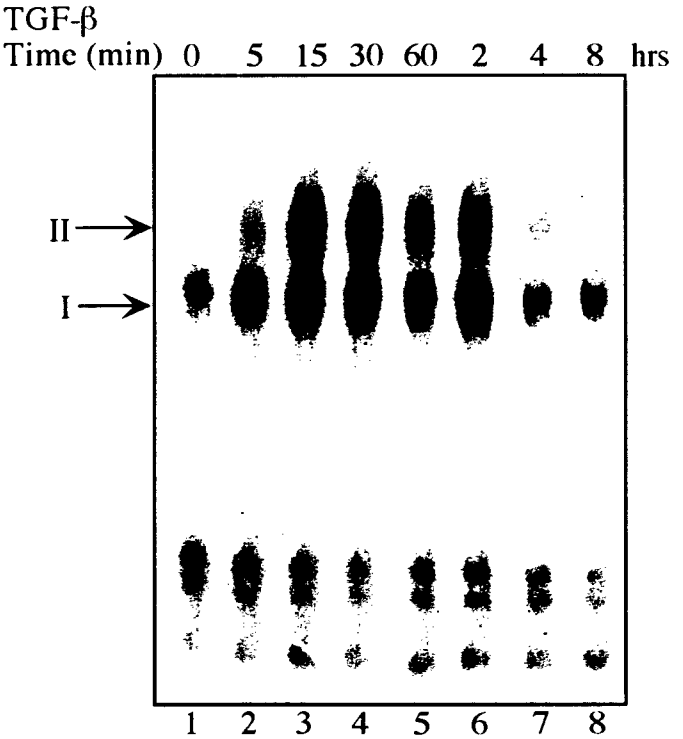
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Fig 5

Figure 5

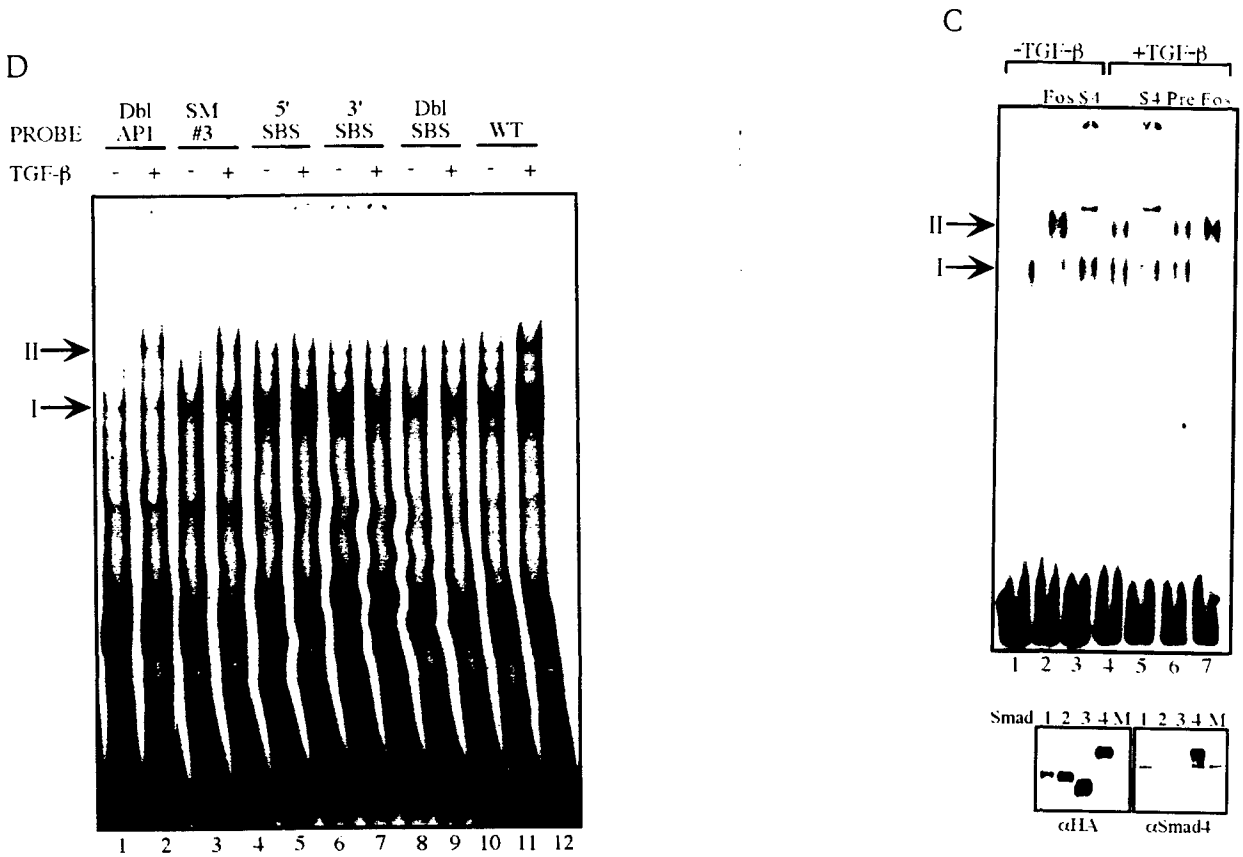
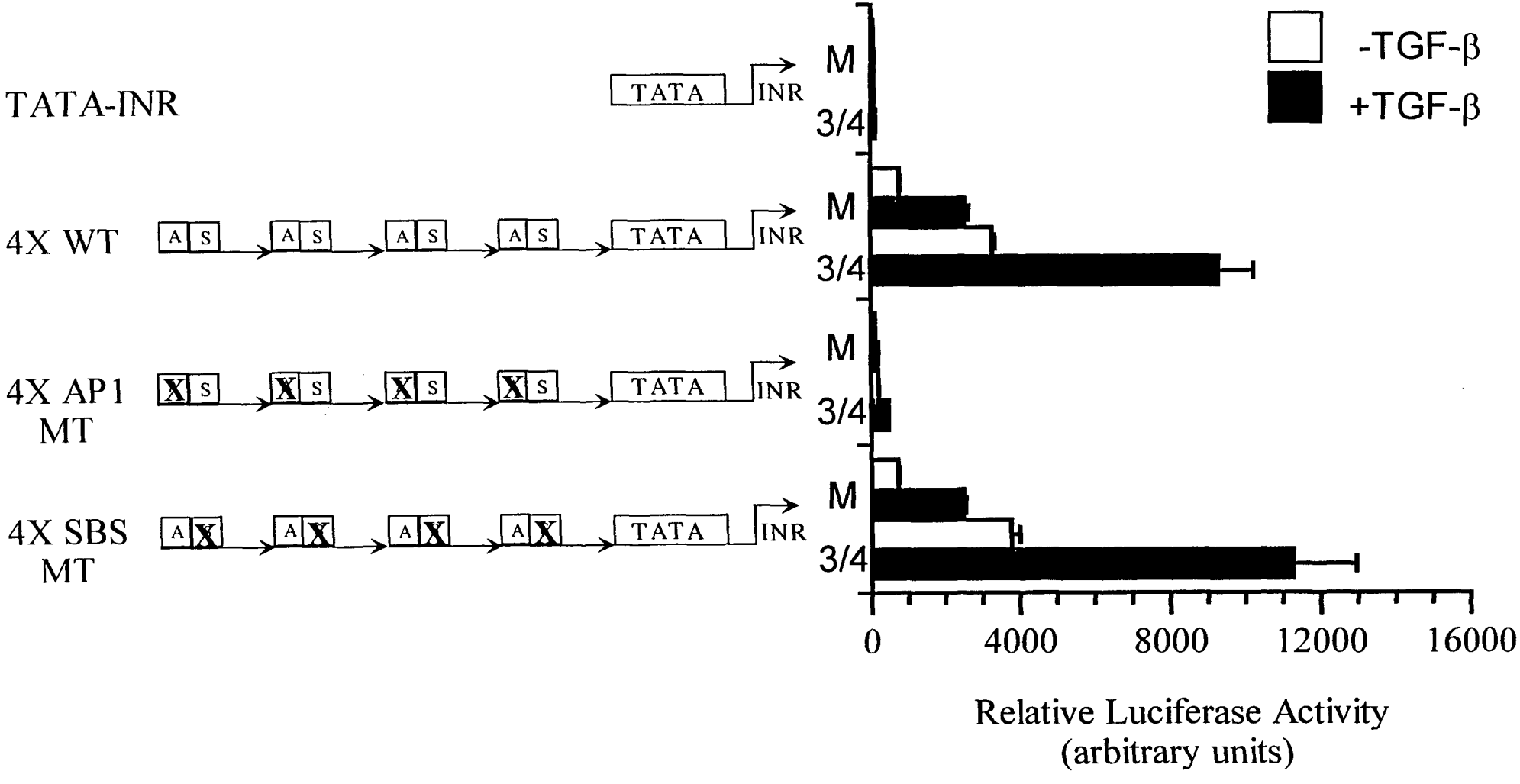


Figure 6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M
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A S
TATA
INR
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4X SBS MT
A X
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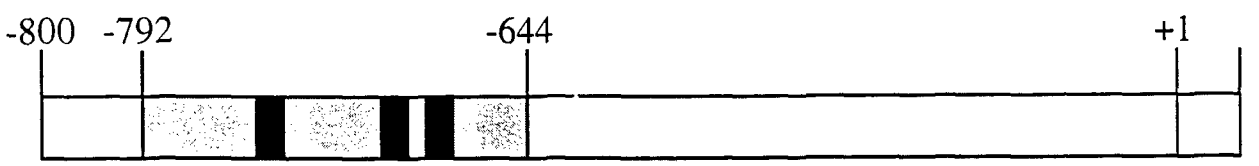
8000
12000
16000
Relative Luciferase Activity
(arbitrary units)

Figure 7

A



PAI-1 promoter region

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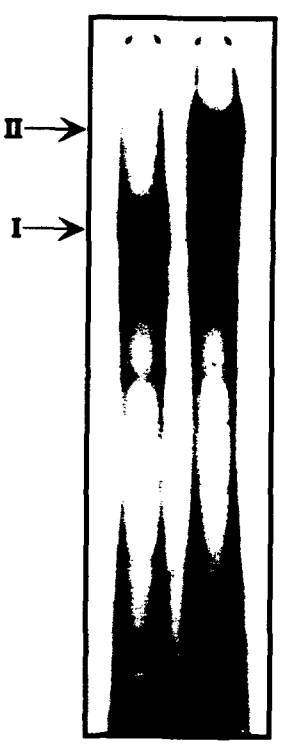

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 19, Issue of May 9, pp. 12862–12867, 1997
Printed in U.S.A.

Expression of Transforming Growth Factor β (TGFβ) Type III Receptor Restores Autocrine TGFβ$_1$ Activity in Human Breast Cancer MCF-7 Cells*

(Received for publication, January 6, 1997, and in revised form, February 20, 1997)

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While transforming growth factor β (TGFβ) type III receptor (RIII) is known to increase TGFβ$_1$ binding to its type II receptor (RII), the significance of this phenomenon is not known. We used human breast cancer MCF-7 cells to study the role of RIII in regulating autocrine TGFβ$_1$ activity because they express very little RIII and no detectable autocrine TGFβ activity. A tetracycline-repressible RIII expression vector was stably transfected into this cell line. Expression of RIII increased TGFβ$_1$ binding to TGFβ type I receptor (RI) as well as RII. Treatment with tetracycline suppressed RIII expression and abolished TGFβ$_1$ binding to RI and RII. Growth of RIII-transfected cells was reduced by 40% when plated at low density on plastic. This reduction was reversed by tetracycline treatment and was partially reversed by treatment with a TGFβ$_1$ neutralizing antibody. The activity of a TGFβ-responsive promoter construct when transiently transfected was more than 3-fold higher in the RIII-transfected cells than in the control cells. Treating the cells with tetracycline or the TGFβ$_1$ neutralizing antibody also significantly attenuated the increased promoter activity. These results suggest that expression of RIII restored autocrine TGFβ$_1$ activity in MCF-7 cells. The RIII-transfected cells were also much less clonogenic in soft agarose than the control cells indicating a reversion of progression. Thus, RIII may be essential for an optimal level of the autocrine TGFβ activity in some cells, especially in the transformed cells with reduced RII expression.

Transforming growth factor β (TGFβ)[1] isoforms are homodimer polypeptides of 25 kDa. They are multifunctional growth factors involved in the regulation of cell proliferation, differentiation, extracellular matrix formation, and immune response (1–3). Many studies have shown that TGFβ can inhibit the growth of a variety cell types including epithelial, endothelial, lymphoid, and myeloid cells (4). Almost all types of cells express one or more of the three isoforms identified in mammals. Although in most systems, the majority of TGFβs secreted is in a latent form with no biological activity, a small percentage can be detected as mature, active TGFβs which may act to regulate cellular functions in an autocrine fashion. For example, neutralization of endogenous TGFβs with anti-TGFβ antibodies was shown to stimulate proliferation of breast and colon cancer cells (5–7). Repression of TGFβ expression by TGFβ1 antisense RNA in colon cancer cells was shown to increase clonogenicity in soft agarose and tumorigenicity in nude mice indicating that autocrine TGFβ activity is tumor-suppressive (8, 9).

TGFβs elicit their effects by binding mainly to three cell surface proteins termed type I (RI), type II (RII), and type III (RIII) receptors. RI and RII are serine/threonine kinase receptors of 55 and 75 kDa, respectively, that form heteromeric complex, apparently at one to one stoichiometric ratio and necessary for TGFβ signal transduction (3). It has been shown that RI requires RII for TGFβ binding, whereas RII needs RI for signaling (10, 11). Recently, several studies showed that mutation or down-regulated expression of RI and/or RII is associated with loss of TGFβ sensitivity and progression of human gastric, colorectal, and prostate cancers and T-cell lymphomas (12–17). Restoration of TGFβ sensitivity by replacing the mutated or down-regulated receptor into colon and breast cancer cells reduced their malignancy (18–20). These results again indicate that autocrine TGFβs can act as negative growth regulators and disruption of the autocrine TGFβ loop is probably a major event contributing to malignant progression.

TGFβ RIII, also called β-glycan, is a proteoglycan of 280–330 kDa. It is the most abundant TGFβ binding molecule on the cell surface of a variety of cell types (21–23). Sequence analyses indicate that human RIII has a relatively small cytoplasmic domain of 41 amino acid residues that contains no consensus signaling motif (23, 24). Therefore, it is believed not to directly transduce TGFβ signal. However, RIII binds all three TGFβ isoforms with high affinity. The K_d ranges from 50 to 300 pM depending on cell types (21, 25, 26). Expression of RIII in the cells that lacks RIII significantly increased TGFβ$_1$ binding to RII by 2.5-fold or more (24, 27). It was shown that in the absence of RIII, only a small population of RII in myoblasts could bind TGFβ$_1$ with high affinity, while a larger population of RII had a much lower affinity for TGFβ$_1$. However, expression of RIII converted all RII receptors into one population with high affinity for TGFβ$_1$ (27). In the presence of ligands, RIII has been shown to form a heteromeric complex with RII suggesting that RIII enhances TGFβ binding to RII by directly presenting the ligands to RII (27, 28).

While it is clear that RIII can enhance TGFβ$_1$ binding to RII, the significance of this phenomenon remains to be elucidated. Since TGFβ isoforms are produced mainly in a latent form and

* This work was supported in part by National Institutes of Health Grant CA63480 and the University of Kentucky Research Fund (to L.-Z. S.) and United States Army Grant DAMD17–94-J-4065 (to X.-F. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TGFβ, transforming growth factor β; RI, RII, and RIII, receptor types I, II, and III, respectively; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

mature, activated TGFβ levels are very low, if detectable, in most cell types, the majority of RII receptors with low affinity would probably not be occupied by the ligands in the absence of RIII. On the basis of what is known about the stoichiometric interaction between TGFβ RI and RII, it appears that RIII should enhance autocrine $TGF\beta_1$ activity if a cell expresses a similar or lower level of RII than RI. Under this condition, autocrine TGFβ activity is minimized without RIII because only a small percentage of RII is ligand-bound and can activate a small percentage of RI to transduce the signal. Thus, expression of RIII should increase the percentage of ligand-bound RII and consequently the percentage of activated RI. To test this hypothesis, we expressed RIII in human breast cancer MCF-7 cells which express a very low level of RII with no detectable autocrine TGFβ activity. We show that expression of RIII inhibited both anchorage-dependent and anchorage-independent growth of MCF-7 cells and the inhibition could be reversed partially by a $TGF\beta_1$ neutralizing antibody, indicating that RIII-induced growth inhibition is at least partially due to restored autocrine negative activity of $TGF\beta_1$.

MATERIALS AND METHODS

Cell Culture—MCF-7 cells were originally obtained from the Michigan Cancer Foundation. The cell line was adapted to McCoy's 5A medium with 10% fetal bovine serum (FBS), pyruvate, vitamins, amino acids, and antibiotics (29). Working cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 and routinely checked for mycoplasma contamination. MCF-7 limiting dilution clones were obtained by plating parental cells into 96-well culture plates at 0.5 cell/well.

RIII Expression Vector Construction and Transfection—The full-length cDNA of rat TGFβ RIII (24) was subcloned by blunt-ended ligation into a tetracycline-repressible expression system as described previously (20). The sense orientation of the RIII cDNA was confirmed by restriction digestion and agarose gel electrophoresis.

The expression vectors were linearized and transfected into one of MCF-7 limiting dilution clones with a BTX Electro Cell Manipulator at 250 V and 950 microfarads. The control cells were transfected with the empty vectors. The transfected cells were plated in 10-cm culture plates and maintained in the 10% FBS medium for 2 days. Selection of stable transfectants were accomplished by adding Geneticin (G418 sulfate; Life Technologies, Inc.) to the culture medium at 600 μg/ml. G418-resistant clones were ring-cloned and expanded for screening of RIII expression. Control clones were pooled and designated as Neo cell.

RNA Analysis—Total RNA from G418-resistant cells was isolated by guanidine thiocyanate homogenization and acidic phenol extraction (30). To measure the transfected rat RIII mRNA levels in the clones, we constructed a rat RIII riboprobe by inserting a 470-base pair *Bam*HI fragment of the rat RIII cDNA into pBSK(−) plasmid (Stratagene Cloning Systems). The recombinant plasmid was then linearized with *Eco*RI and T_3 RNA polymerase was used to synthesize a radioactive antisense RIII probe. RNase protection assays were performed using this radioactive antisense riboprobe to measure RIII mRNA levels in the transfected clones as described previously (20).

Receptor Cross-linking—Simian recombinant $TGF\beta_1$ was purified from conditioned media of transfected Chinese hamster ovary cells as described previously (31). Purified $TGF\beta_1$ was iodinated by the chloramine T method as described by Ruff and Rizzino (32). To measure the expression of cell surface TGFβ receptors, [^{125}I]$TGF\beta_1$ (200 pM) was incubated with a cell monolayer in 35-mm tissue culture wells and then cross-linked to its receptors as described by Segarini *et al.* (33). Labeled cell monolayers were solubilized in 200 μl of 1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride. Equal amounts of cell lysate protein were electrophoresed in 4–10% gradient SDS-polyacrylamide gel electrophoresis under reducing conditions and exposed for autoradiography.

Measurement of Secreted Mature $TGF\beta_1$—To measure the amount of active $TGF\beta_1$ in the media conditioned by control or RIII-transfected cells, monolayer cells in 6-well culture plates were cultured in the 10% FBS medium until confluence. The cells were washed twice with a serum-free McCoy's 5A medium and incubated in 1.5 ml of this serum-free medium for additional 72 h. The conditioned medium was then collected in siliconized microcentrifuge tubes and the cell number was counted with a hemocytometer. The amount of mature, activated $TGF\beta_1$ in the conditioned medium was determined with a $TGF\beta_1$ ELISA kit from Promega (Madison, WI) according to the manufacturer's instructions.

Plating Efficiency Assay—To study the effect of RIII expression on cell proliferation at low seeding densities, the control and RIII-transfected cells were plated at 200 or 400 cells per well in 24-well culture plates. After 9 days of incubation, relative cell number was determined with an MTT assay as described previously (20). To determine whether autocrine $TGF\beta_1$ activity was enhanced after RIII expression, a $TGF\beta_1$ neutralizing antibody (R&D Systems) was added to the cells after plating at a final concentration of 30 μg/ml.

Transient Transfection and Luciferase Assay—To determine whether RIII expression enhanced autocrine $TGF\beta_1$ activity, we measured a TGFβ-responsive promoter activity using a plasmid called p3TP-Lux from Dr. J. Massague. The promoter activity is reported by luciferase (10). The p3TP-Lux (20 μg) and a β-galactosidase expression plasmid (5 μg) were transiently co-transfected into the MCF-7 Neo control or RIII-expressing cells (10^7 cells) by electroporation in the same manner as the stable transfection described above. The cells were then equally divided into replicate culture dishes, part of which were treated with tetracycline (0.1 μg/ml) or the $TGF\beta_1$ neutralizing antibody (30 μg/ml) for 48 h. The treated and control cells were lysed in luciferase buffer (100 mM K_2HPO_4, pH 7.8, 1 mM dithiothreitol) using three cycles of freeze-thaw. The activities of luciferase and β-galactosidase in the cell extract were assayed using published procedures (34, 35). Luciferase activity was normalized to β-galactosidase activity and expressed as relative luciferase activity.

Soft Agarose Assay—To compare the clonogenic potential of the control and RIII-transfected cells in a semisolid medium, soft agarose assays were performed as described previously (20). Briefly, 6×10^3 cells were suspended in 1 ml of 0.4% low melting point agarose (Life Technologies) dissolved in the 10% FBS medium and plated on the top of a 1-ml underlayer of 0.8% agarose in the same medium in 6-well culture plates. For tetracycline treatment, we added tetracycline in soft agarose at 0.1 μg/ml as well as 200 μl of the medium containing 0.2 μg/ml tetracycline on the top of the solidified agarose. Control wells received 200 μl of the medium without tetracycline. The media were replenished every 3 days. After 3 weeks of incubation in the humidified incubator with 5% CO_2 at 37 °C, the cell colonies were visualized by staining with 1 ml of *p*-iodonitrotetrazolium violet staining (Sigma). To quantitate the clonogenicity, the colonies in each well were counted under a magnifying lens.

RESULTS

Expression of RIII—MCF-7 cells express very low levels of RIII and undetectable RII on the cell surface using the receptor cross-linking method (20). However, they express low levels of RII mRNA and their growth can be slightly inhibited by high concentrations of exogenous $TGF\beta_1$ suggesting that they do have functional TGFβ receptors and intracellular signaling components (20). In addition, this cell line expresses mainly $TGF\beta_1$ with no detectable $TGF\beta_2$ and $TGF\beta_3$ (36).[2] Therefore, this cell line is ideal for the examination of whether TGFβ RIII plays a role in regulating autocrine $TGF\beta_1$ activity and, consequently, cell growth.

Tetracycline-repressible RIII expression vectors were transfected into one of the MCF-7 limiting dilution clones. Using RNase protection assays and a rat RIII riboprobe, we identified two RIII-expressing clones. Both clones showed similar levels of RIII expression and growth properties on plastic and in soft agarose (data not shown). Therefore, we used one clone for the study. The mRNA level of the transfected RIII in this clone, designated as RIII cell, is shown in Fig. 1. Consistent with the mRNA level, RIII cells also expressed a higher level of cell surface RIII protein than the Neo cells by the receptor cross-linking assay (Fig. 2). The specificity of [^{125}I]$TGF\beta_1$ binding to the receptor was confirmed by competition with a 100-fold excess of unlabeled $TGF\beta_1$ (Fig. 2, *third lane*). Treatment with tetracycline at 0.1 μg/ml for 4 days prior to the receptor cross-linking assay almost completely suppressed the expression of transfected RIII (Fig. 2, *fourth lane*). This reversible expression

[2] C. Chen and L.-Z. Sun, unpublished observations.

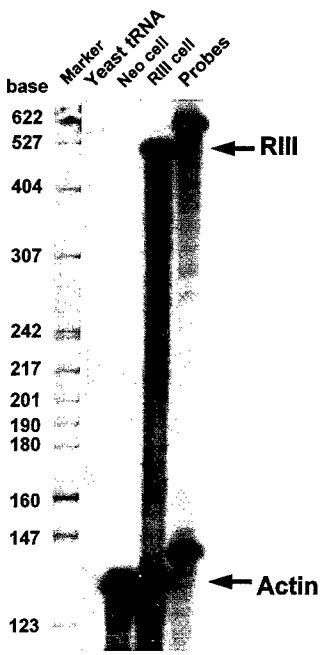

FIG. 1. **Expression of TGFβ RIII mRNA in the MCF-7 transfectants.** A typical MCF-7 limiting dilution clone was stably transfected with rat RIII expression plasmids and selected with Geneticin. The mRNA of the transfected RIII was detected in 20 μg of total RNA from an RIII-expressing clone by an RNase protection assay with a rat RIII riboprobe. The control cells were transfected with the plasmids without RIII cDNA and designated as Neo cells. Yeast tRNA was used as a negative control for the assay. Human actin mRNA levels were used for normalization.

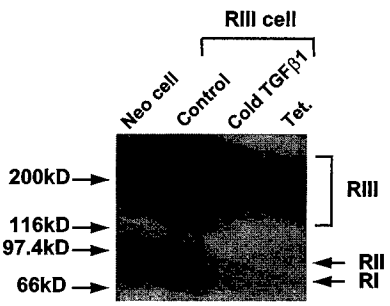

FIG. 2. **Cell surface expression of TGFβ receptors in the MCF-7 transfectants.** Receptor cross-linking assays were used to examine the cell surface profiles of the TGFβ receptors in the Neo and RIII cells. Confluent monolayer cultures of the Neo and RIII cells were incubated with 200 pM ^{125}I-TGFβ$_1$ alone (*first* and *second lanes*) or in the presence of 20 nM cold TGFβ$_1$ (*third lane*) for 3 h at 4 °C. For the *fourth lane*, the RIII cells were cultured in the presence of 0.1 μg/ml tetracycline for 4 days prior to the receptor cross-linking assay. The receptor-bound ^{125}I-TGFβ$_1$ was cross-linked with disuccinimidyl suberate. Cell lysates containing equal amounts of protein were electrophoresed in 4–10% gradient SDS-polyacrylamide gel electrophoresis under reducing conditions. The ^{125}I-TGFβ$_1$-linked receptors were visualized after autoradiography.

of RIII by tetracycline was particularly useful in the subsequent experiments for the demonstration of the specific effect of the RIII on the cell growth and autocrine TGFβ activity.

Consistent with a previous observation (20), TGFβ RI and RII were not detectable by the receptor cross-linking assay on the Neo cell surface (Fig. 2). However, two bands corresponding to RI and RII were detected in the RIII cell samples. Since they were absent in the Neo cells and in the tetracycline-treated RIII cells (Fig. 2), the increased TGFβ$_1$ binding to RI and RII appears to be specifically due to RIII expression. This is consistent with what was observed in myoblasts (24, 27).

Expression of Endogenous TGFβ$_1$—While RIII cells showed

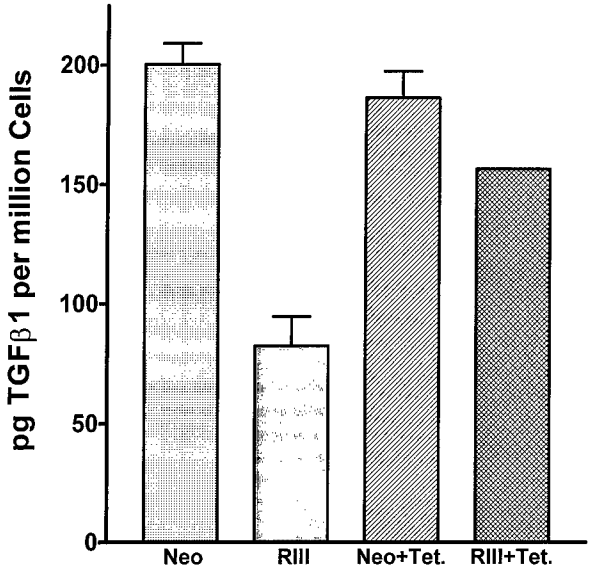

FIG. 3. **Activated TGFβ$_1$ in the conditioned media of the MCF-7 transfectants.** The Neo and RIII cells in 6-well plates were cultured in the 10% FBS medium with or without 0.1 μg/ml tetracycline (*Tet.*) till confluence. The medium was then changed to a serum-free McCoy's 5A medium with or without tetracycline and incubated for 72 h. The conditioned media were collected from duplicate wells. Activated TGFβ$_1$ in the media was determined as described under "Materials and Methods." The amount of activated TGFβ$_1$ is normalized by the cell number and expressed as the mean ± S.E. of four measurements except the mean of RIII+Tet. which was the average of two measurements with identical values.

increased TGFβ$_1$ binding to RI and RII, their sensitivity to exogenous TGFβ$_1$ remained relatively similar to that of the Neo cells in a growth inhibition assay (20) (data not shown). Therefore, we hypothesized that MCF-7 cells may express enough activated TGFβ$_1$ such that the expression of RIII regenerated a maximal TGFβ growth inhibition in an autocrine manner. To test this hypothesis, we first examined whether the cells produced mature, activated TGFβ$_1$ in our system. Using a TGFβ$_1$ ELISA kit we were able to detect a modest amount of activated TGFβ$_1$ (200 pg/10^6 cells/72 h) in the medium conditioned by the Neo cells. The amount of activated TGFβ$_1$ in the medium conditioned by the RIII cells was reduced by more than half (Fig. 3). This reduction could be reversed by treating the RIII cells with tetracycline, suggesting that the reduced amount of free activated TGFβ$_1$ in the medium was due to its binding to RIII. This result led us to further examine whether the cell growth was inhibited by the autocrine TGFβ$_1$ after RIII expression.

Plating Efficiency Assay—It is well known that TGFβ isoforms suppress cell cycle progression of newly plated, nondividing cells more effectively than that of exponentially growing cells. Plating cells at low density is one way to ensure that the cells are maintained for a period of non- or slow-dividing state before entering exponential growth. This approach has been successfully employed previously to demonstrate the autocrine inhibitory activity of TGFβ (18, 19). When Neo and RIII cells were plated in 24-well tissue culture plates at 200 and 400 cells/well, the RIII cells proliferated significantly slower than the Neo cells by about 40% after nine days of incubation (Fig. 4*A*). The growth reduction was due to reduced number of colonies as well as number of cells per colony. Treatment of the cells with tetracycline at 0.1 μg/ml prior to and during the plating efficiency assay completely reversed the growth property of the RIII cells back to that of the Neo cells as shown in Fig. 4*B* suggesting that the growth inhibition was specifically due to RIII expression. To assess whether the growth reduction

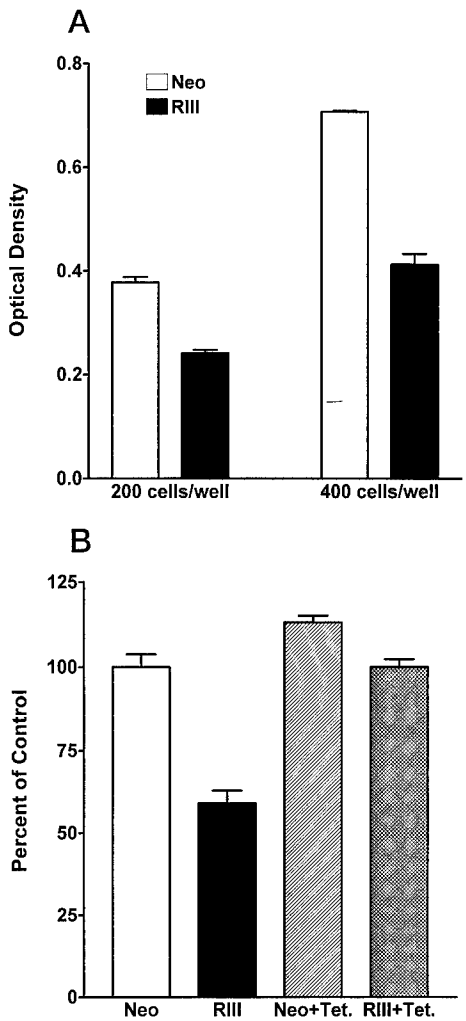

FIG. 4. **Plating efficiency of the MCF-7 transfectants.** *A*, the Neo and RIII cells were plated in a 24-well plate at 200 or 400 cells/well in the 10% FBS medium and incubated for 9 days. The relative cell numbers were determined with the MTT assay and expressed as optical density values at 590 nm. *B*, the Neo and RIII stock cells were first cultured in the absence or presence of 0.1 μg/ml tetracycline (*Tet.*) for 1 week and then plated in a 24-well plate at 400 cells/well in the 10% FBS medium with or without 0.1 μg/ml tetracycline. The media were changed every other day to replenish the tetracycline. The MTT assay was performed after 6 days of incubation and the OD values of each treatment were expressed as percent of the optical density value of the Neo cells. The values in both *panels A* and *B* are presented as means ± S.E. of six OD measurements from duplicate wells.

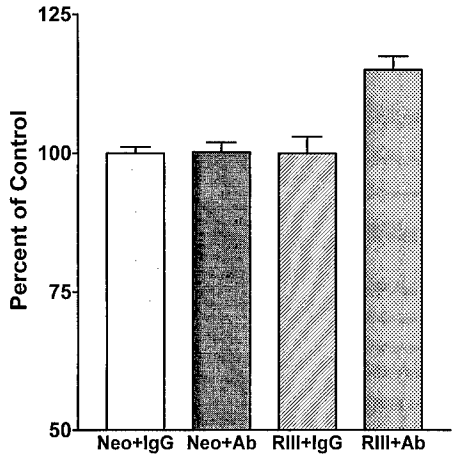

FIG. 5. **Effect of a TGFβ$_1$ neutralizing antibody on the plating efficiency of the MCF-7 transfectants.** The Neo and RIII cells were plated in 24-well plates at 400 cells/well in the presence of 30 μg/ml normal IgG (IgG) or 30 μg/ml TGFβ$_1$ neutralizing antibody (*Ab*). The MTT assay was performed after 9 days of incubation. The OD values of TGFβ$_1$ antibody treatment are presented as percent of the respective IgG-treated controls. The values are means ± S.E. determined in 6 samples from duplicate wells.

was due to RIII-enhanced autocrine TGFβ$_1$ activity, we treated the cells with a TGFβ$_1$ neutralizing antibody or a control antibody after plating. While the growth of Neo cells was not affected by the antibody, the growth of the RIII cells was stimulated (Fig. 5). Even though the stimulation was only 15%, it is statistically significant ($p < 0.05$) by Student's *t* test. Since RIII expression reduced the growth by 40%, 15% growth stimulation represents a 37.5% recovery of the growth. The reason that tetracycline was more effective than the TGFβ$_1$ neutralizing antibody to stimulate the growth of the RIII cells was probably due to the fact that tetracycline could completely suppress RIII expression whereas the antibody had to compete with the RIII for endogenous TGFβ$_1$.

Activity of a TGFβ-responsive Promoter—To confirm our observation that RIII expression enhanced autocrine TGFβ$_1$ activity, we measured the activity of a TGFβ-responsive promoter/luciferase construct after being transiently transfected into the Neo and RIII cells. This construct has been widely used to measure the efficacy of TGFβ in activating its signaling receptors and stimulating gene expression (10). While RIII expression inhibited the growth of the MCF-7 cells, it stimulated TGFβ-responsive promoter activity as indicated by luciferase activity by more than 3-fold (Fig. 6*A*). Treatment of the transiently transfected cells with tetracycline for 48 h reduced the luciferase activity almost to the level of the Neo cells, again demonstrating the specificity of the RIII effect. Similar to the growth assay, the stimulated promoter activity could be partially, but significantly ($p < 0.05$) reduced by treating the transfected RIII cells with the TGFβ$_1$ neutralizing antibody for 48 h. Thus, with two different approaches, we have showed that RIII can restore autocrine TGFβ$_1$ activity in the MCF-7 cells.

Clonogenicity in Soft Agarose—The growth inhibitory activity of autocrine TGFβ is known to maintain cancer cells at a less malignant state as previously shown by its suppressive effect on the clonogenicity in soft agarose and the tumorigenicity in nude mice (8, 18, 20). Since RIII expression restored autocrine TGFβ$_1$ activity, we compared the ability of Neo and RIII cells to form colonies in a soft agarose assay. As shown in Fig. 7*A*, the number of colonies formed by RIII cells were considerably less than that formed by Neo cells. Tetracycline had no effect on Neo cells, but significantly increased the number of colonies formed by RIII cells (Fig. 7*B*). These results suggest that by restoring autocrine TGFβ$_1$ activity, RIII can also reduce the anchorage-independent growth of the MCF-7 cells in the same manner as RII (20).

DISCUSSION

Although RIII has been shown to enhance TGFβ$_1$ binding to RII, this is the first report demonstrating an important role of RIII in regulating autocrine TGFβ$_1$ activity. Expression of RIII in MCF-7 cells increased affinity labeling of both RI and RII by [^{125}I]TGFβ$_1$ to a detectable level. Increased TGFβ$_1$ binding to RII after RIII expression is consistent with what was observed in myoblasts (24, 27). However, RIII expression in myoblasts had no effect on TGFβ$_1$ binding to RI. The authors suspected that the amount of RII with high affinity for TGFβ$_1$ in the myoblasts without RIII was sufficiently high enough to support TGFβ$_1$ binding to all cell surface RI molecules (27). In contrast, MCF-7 cells expressed a very low level of RII mRNA and no detectable amount of cell surface RII protein with the affinity labeling (20). As a result, even though they express a relatively

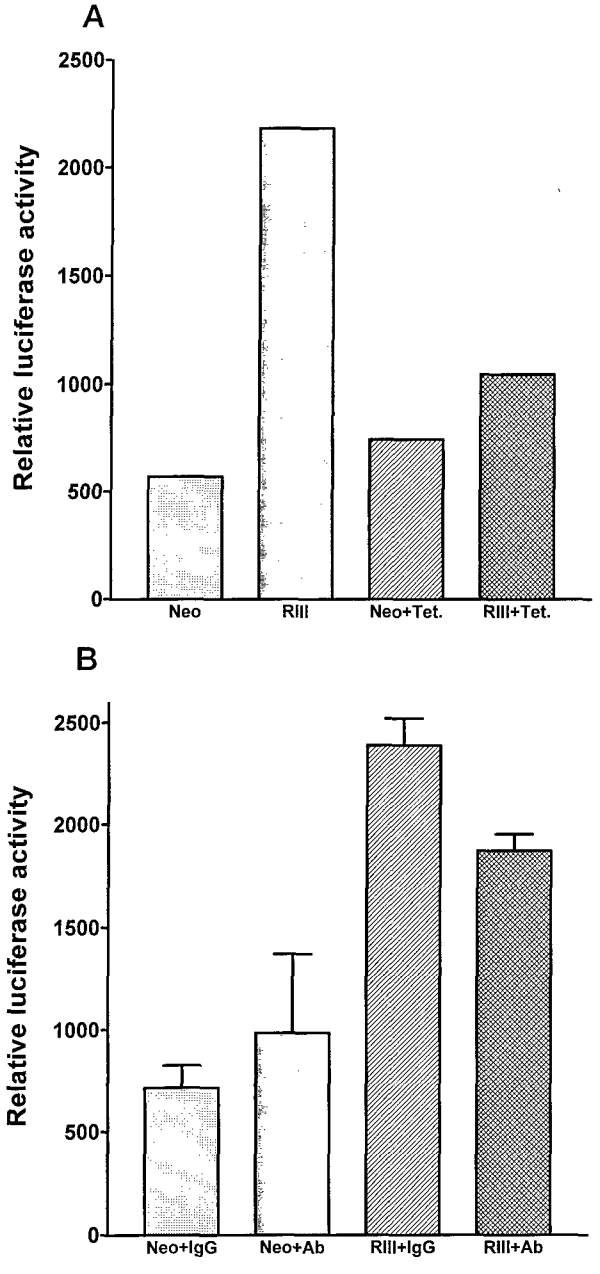

FIG. 6. **The activity of a TGFβ-responsive promoter in the MCF-7 transfectants.** The Neo and RIII cells were transiently co-transfected with p3TP-Lux and a β-galactosidase expression plasmid by electroporation. Then, the transfected cells were equally divided into two (*panel A*) or six (*panel B*) 35-mm culture wells. In *panel A*, one of the two wells was treated with 0.1 μg/ml tetracycline (*Tet.*) during a 48-h incubation. In *panel B*, three of the six wells were treated with 30 μg/ml normal IgG (IgG), whereas the other three wells were treated with 30 μg/ml $TGF\beta_1$ neutralizing antibody (*Ab*) during a 48-h incubation. The cells were harvested 48 h after transfection. The activities of the luciferase and β-galactosidase in the cell lysate were separately measured. The data are presented as relative luciferase activity after normalized to the β-galactosidase activity. The experiment presented in *panel A* was repeated with similar results. The values in *panel B* are presented as means ± S.E. of three replicate wells.

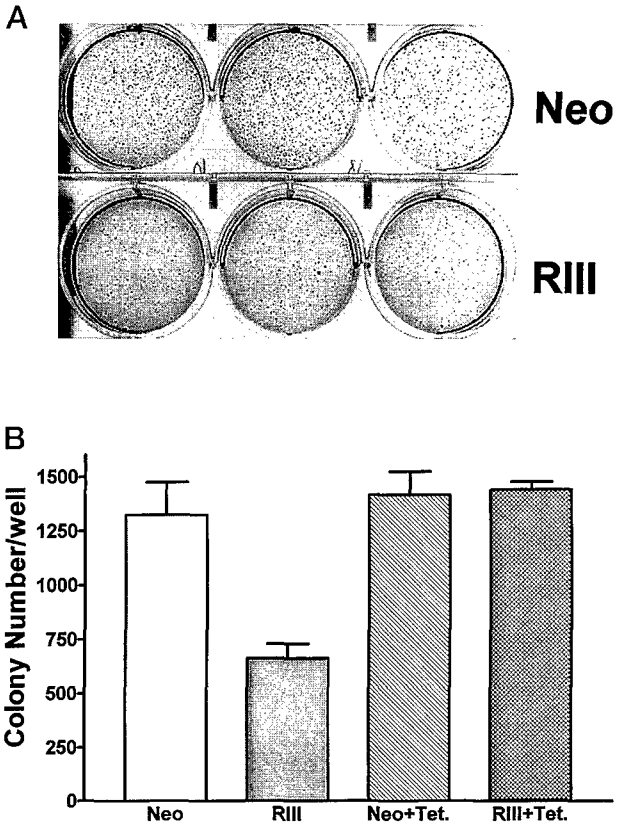

FIG. 7. **Anchorage-independent colony formation in soft agarose of the MCF-7 transfectants.** Exponentially growing Neo and RIII cells (6,000 cells) were resuspended in 1 ml of 0.4% low melting point agarose in the 10% FBS medium and plated on top of a 1-ml underlayer of 0.8% agarose in a 6-well culture plate. Tetracycline treatment was carried out as described under "Materials and Methods." After 3 weeks of incubation, cell colonies were visualized by staining with 1 ml of *p*-iodonitrotetrazolium violet (*panel A*). The number of colonies were counted in three wells for each treatment and presented as means ± S.E. (*panel B*).

high level of RI mRNA (20), little RI could be affinity-labeled with $TGF\beta_1$ since RI requires TGFβ-bound RII for ligand binding. RIII expression in MCF-7 cells had no effect on RII mRNA expression,[2] suggesting that the increased $TGF\beta_1$ binding to RII in the RIII cells was due to increased binding affinity as observed in the myoblasts (27). It appears that the increased $TGF\beta_1$ binding to RI resulted from the increased $TGF\beta_1$ binding to RII. Therefore, whether RIII is necessary for maintaining an optimal autocrine $TGF\beta_1$ activity in a given cell type appears to be dependent on the ratio of RII to RI.

In the myoblasts that lack RIII expression, expression of RIII was shown to convert RII molecules into a single population with high affinity (27). Due to its low expression level, we do not know whether the two populations of RII also exist in MCF-7 cells. The fact that their growth can only be inhibited by high concentrations of exogenous $TGF\beta_1$ (20) suggests that they contain the low affinity RII. Since the $TGF\beta_1$ neutralizing antibody had no effect on the growth of the Neo cells, it appears that they contain no or little high affinity RII which does not confer a significant amount of autocrine $TGF\beta_1$ activity.

TGFβ RI, RII, and RIII are widely expressed in many types of normal and transformed cells (37). Whether RIII plays a role in regulating autocrine TGFβ activity in certain types of normal cells remains to be elucidated. However, our data would suggest that if a normal cell expresses less RII than RI, RIII could be essential to confer TGFβ sensitivity. This could also be the case during development of a cell or an organ when the ratio of RII to RI may vary at different stages. In transformed cells including breast, colon, stomach, prostate cancer cells, retinoblastoma cells, and lymphoma cells, RII is often down-regulated (12–14, 17, 38–42). While in some cancers such as colon and gastric cancers, the down-regulation of RII can be due to gene mutation (12, 14, 15), in other cases, it may be due to reduced gene expression as in the MCF-7 cells. If the latter is true for a given type of transformed cells, RIII level will be critical to the maintenance of the autocrine TGFβ activity and

consequently a less malignant phenotype of the cells. The fact that the expression of RIII shown in this report can be as effective as the expression of a moderate level of RII (20) in suppressing the anchorage-independent growth of the MCF-7 cells demonstrates this important role of RIII in keeping the autocrine $TGF\beta_1$ activity at an optimal level. Since $TGF\beta_3$ behaves similarly to $TGF\beta_1$ in receptor binding and $TGF\beta_2$ requires RIII for binding to the signaling receptors (27), it is conceivable that RIII can also be critical to the maintenance of the autocrine activities of $TGF\beta_2$ and $TGF\beta_3$ in the cells that express these two isoforms.

While exogenous TGFβ can inhibit the growth of many types of transformed cells (4), studies have shown that in some systems, autocrine TGFβ can render cells insensitive to the growth inhibitory activity of exogenous $TGF\beta_1$ (18). This is likely due to the fact that the cells not only produce high levels of one or more TGFβ isoforms, but also can activate them. In $TGF\beta_1$-transfected fibroblasts and fibrosarcoma cells, it was found that a significant amount of cell-activated $TGF\beta_1$ was bound to the cell surface even though very little activated $TGF\beta_1$ could be detected in the conditioned medium (43). The MCF-7 cells we used produced a readily detectable amount of mature, activated $TGF\beta_1$ in the conditioned medium as shown in Fig. 3. In addition, the cells express a low level of RII which should be easily saturable by the ligand. Therefore, we hypothesized that although RIII-enhanced $TGF\beta_1$ binding to the signaling receptor RII, the reason that the sensitivity of the MCF-7 cells to the growth inhibitory activity of the exogenous $TGF\beta_1$ was not increased after RIII expression was due to the fact that the autocrine $TGF\beta_1$ had generated a maximal level of growth inhibition by saturating RII and/or the intracellular signaling pathway. Since RIII is known to form a heteromeric complex with RII in the presence of activated TGFβ (27, 28), its main role is probably to make the RII more accessible by the ligand. In addition, by capturing and retaining ligand, RIII may protect the ligand from cellular degradation and/or inactivation, rendering more ligand available for binding to the signaling receptors. The RIII-enhanced autocrine $TGF\beta_1$ activity was demonstrated in the MCF-7 cells by the fact that a $TGF\beta_1$ neutralizing antibody could partially reverse the RIII-induced growth inhibition and gene expression.

Because of its short cytoplasmic domain with no consensus signaling motif and its absence in some of the TGFβ-sensitive cells, RIII has been regarded as a non-signaling, accessory TGFβ receptor. As a result, the RIII-generated growth inhibition we observed in MCF-7 cells was solely attributed to the increased autocrine $TGF\beta_1$ binding to RII and, consequently the activation of RI·RII receptor complex even though $TGF\beta_1$ neutralizing antibody only abrogated about one-third of the RIII-induced growth inhibition as well as gene expression. However, our study cannot rule out the possibility that RIII may transduce signal by itself or in association with RII. We are currently attempting to address this possibility.

In summary, our study showed that the expression of RIII can lead to the inhibition of both anchorage-dependent and anchorage-independent growth in MCF-7 cells. This is at least in part mediated by RIII-restored autocrine inhibitory activity of the endogenous $TGF\beta_1$. RIII may be essential for an optimal level of autocrine TGFβ activity in various types of normal cells. Its expression is likely to be critical for the maintenance of the growth inhibitory activity of autocrine TGFβ isoforms and, consequently of a less malignant phenotype in other adenocarcinoma cells with reduced RII expression.

Acknowledgments—We thank Dr. H. Bujard at the University of Heidelberg, Germany, for the tetracycline-repressible expression plasmids and Dr. J. Massague at Memorial Sloan-Kettering Cancer Center, New York, for the p3TP-Lux plasmid. We also thank Dr. David Kaetzel at the University of Kentucky College of Medicine for critical reading of this manuscript.

REFERENCES

1. Moses, H. L., Yang, E. Y., and Pietenpol, J. A. (1990) *Cell* **63,** 245–247
2. Roberts, A. B. and Sporn, M. B. (1991) in *Peptide Growth Factors and Their Receptors* (Sporn, M. B., and Roberts, A. B., eds) pp. 419–472, Springer-Verlag, Heidelberg
3. Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massague, J. (1994) *Biochim. Biophys. Acta* **1222,** 71–80
4. Lyons, R. M., and Moses, H. L. (1990) *Eur. J. Biochem.* **187,** 467–473
5. Arteaga, C. L., Coffey, R. J., Jr., Dugger, T. C., McCutchen, C. M., Moses, H. L., and Lyons, R. M. (1990) *Cell Growth Differ.* **1,** 367–374
6. Hafez, M. M., Infante, D., Winawer, S., and Friedman, E. (1990) *Cell Growth Differ.* **1,** 617–626
7. Sun, L., Wu, S., Coleman, K., Fields, K. C., Humphrey, L. E., and Brattain, M. G. (1994) *Exp. Cell Res.* **214,** 215–224
8. Wu, S. P., Theodorescu, D., Kerbel, R. S., Willson, J. K., Mulder, K. M., Humphrey, L. E., and Brattain, M. G. (1992) *J. Cell Biol.* **116,** 187–196
9. Wu, S. P., Sun, L. Z., Willson, J. K., Humphrey, L., Kerbel, R., and Brattain, M. G. (1993) *Cell Growth Differ.* **4,** 115–123
10. Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massague, J. (1992) *Cell* **71,** 1003–1014
11. Vivien, D., Attisano, L., Wrana, J. L., and Massague, J. (1995) *J. Biol. Chem.* **270,** 7134–7141
12. Park, K., Kim, S. J., Bang, Y. J., Park, J. G., Kim, N. K., Roberts, A. B., and Sporn, M. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8772–8776
13. Kadin, M. E., Cavaille-Coll, M. W., Gertz, R., Massague, J., Cheifetz, S., and George, D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6002–6006
14. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M., and Willson, J. K. V. (1995) *Science* **268,** 1336–1338
15. Myeroff, L. L., Parsons, R., Kim, S. J., Hedrick, L., Cho, K. R., Orth, K., Mathis, M., Kinzler, K. W., Lutterbaugh, J., Park, K., Bang, Y. J., Lee, H. Y., Park, J. G., Lynch, H. T., Roberts, A. B., Vogelstein, B., and Markowitz, S. D. (1995) *Cancer Res.* **55,** 5545–5547
16. Kim, I. Y., Ahn, H. J., Zelner, D. J., Shaw, J. W., Sensibar, J. A., Kim, J. H., Kato, M., and Lee, C. (1996) *Cancer Res.* **56,** 44–48
17. Kim, I. Y., Ahn, H. J., Zelner, D. J., Shaw, J. W., Lang, S., Kato, M., Oefelein, M. G., Miyazono, K., Nemeth, J. A., Kozlowski, J. M., and Lee, C. (1996) *Clin. Cancer Res.* **2,** 1255–1261
18. Wang, J., Sun, L., Myeroff, L., Wang, X., Gentry, L. E., Yang, J., Liang, J., Zborowska, E., Markowitz, S., Willson, J. K. V., and Brattain, M. G. (1995) *J. Biol. Chem.* **270,** 22044–22049
19. Wang, J., Han, W., Zborowska, E., Liang, J., Wang, X., Willson, J. K. V., Sun, L., and Brattain, M. G. (1996) *J. Biol. Chem.* **271,** 17366–17371
20. Sun, L., Wu, G., Willson, J. K. V., Zborowska, E., Yang, J., Rajkarunanayake, I., Wang, J., Gentry, L. E., Wang, X.-F., and Brattain, M. G. (1994) *J. Biol. Chem.* **269,** 26449–26455
21. Cheifetz, S., Bassols, A., Stanley, K., Ohta, M., Greenberger, J., and Massague, J. (1988) *J. Biol. Chem.* **263,** 10783–10789
22. Segarini, P. R., Rosen, D. M., and Seyedin, S. M. (1989) *Mol. Endocrinol.* **3,** 261–272
23. Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S., and Massague, J. (1991) *Cell* **67,** 785–795
24. Wang, X. F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F., and Weinberg, R. A. (1991) *Cell* **67,** 797–805
25. Cheifetz, S., Hernandez, H., Laiho, M., ten Dijke, P., Iwata, K. K., and Massague, J. (1990) *J. Biol. Chem.* **265,** 20533–20538
26. Andres, J. L., Ronnstrand, L., Cheifetz, S., and Massague, J. (1991) *J. Biol. Chem.* **266,** 23282–23287
27. Lopez-Casillas, F., Wrana, J. L., and Massague, J. (1993) *Cell* **73,** 1435–1444
28. Moustakas, A., Lin, H. Y., Henis, Y. I., Plamondon, J., O'Connor-McCourt, M. D., and Lodish, H. F. (1993) *J. Biol. Chem.* **268,** 22215–22218
29. Mulder, K. M., and Brattain, M. G. (1989) *Mol. Endocrinol.* **3,** 1215–1222
30. Xie, W. Q., and Rothblum, L. I. (1991) *Biotechniques* **11,** 324, 326–327
31. Gentry, L. E., Lioubin, M. N., Purchio, A. F., and Marquardt, H. (1988) *Mol. Cell Biol.* **8,** 4162–4168
32. Ruff, E., and Rizzino, A. (1986) *Biochem. Biophys. Res. Commun.* **138,** 714–719
33. Segarini, P. R., Roberts, A. B., Rosen, D. M., and Seyedin, S. M. (1987) *J. Biol. Chem.* **262,** 14655–14662
34. Schmidt, J. V., Carver, L. A., and Bradfield, C. A. (1993) *J. Biol. Chem.* **268,** 22203–22209
35. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
36. Arrick, B. A., Korc, M., and Derynck, R. (1990) *Cancer Res.* **50,** 299–303
37. Massague, J. (1990) *Annu. Rev. Cell Biol.* **6,** 597–641
38. Kimchi, A., Wang, X. F., Weinberg, R. A., Cheifetz, S., and Massague, J. (1988) *Science* **240,** 196–199
39. Arteaga, C. L., Tandon, A. K., Von Hoff, D. D., and Osborne, C. K. (1988) *Cancer Res.* **48,** 3898–3904
40. Kalkhoven, E., Roelen, B. A., de Winter, J. P., Mummery, C. L., van den Eijnden-van Raaij, A. J., van der Saag, P. T., and van der Burg, B. (1995) *Cell Growth Differ.* **6,** 1151–1161
41. Hoosein, N. M., McKnight, M. K., Levine, A. E., Mulder, K. M., Childress, K. E., Brattain, D. E., and Brattain, M. G. (1989) *Exp. Cell Res.* **181,** 442–453
42. Filmus, J., Zhao, J., and Buick, R. N. (1992) *Oncogene* **7,** 521–526
43. Beauchamp, R. D., Sheng, H. M., Bascom, C. C., Miller, D. A., Lyons, R. M., Torre-Amione, G., and Moses, H. L. (1992) *Endocrinology* **130,** 2476–2486